Regulation of the *Saccharomyces cerevisiae* DNA Replication Protein Cdc6p by the Cyclin Dependent Kinases

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This thesis is dedicated to my parents,

Thomas and Patricia Elsasser,

for teaching me tenacity.

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ABSTRACT

A key feature of DNA replication in higher eukaryotes is that duplication of the genome occurs by DNA replication beginning at sequence-specific sites on the chromatin, and proceeds in each direction from this site of initiation. The DNA replication forks migrate toward each other but do not overlap. Furthermore, origin firing is restricted to one phase per cell cycle, and the cell regulates this by only allowing origin priming within a small window of the cell cycle. DNA replication, and cell cycle transitions in general, are governed by the cyclin dependent kinases, which are comprised of various cyclins associated with Cdk1p. The regulation of the *Saccharomyces cerevisiae* DNA replication protein Cdc6p by the cyclin dependent kinases is the subject of this thesis.

During the purification of Cdc6p from *Saccharomyces cerevisiae,* the Cdk1p was found to form a tight complex with Cdc6p. Since Cdc6p had previously been shown to be required for the initiation of DNA replication, and a subset of the cyclin dependent kinases had also been shown to modulate this event, this interaction was characterized. Cdc6p was found to interact with and be phosphorylated only by Cdk1p complexes associated with B-type cyclins. The B-type cyclins have been shown to have two roles in the initiation of DNA replication: one is triggering the firing of DNA replication origins at the G1/S transitions, and the other is blocking the subsequent priming of origins until the beginning of the next cell cycle. The interaction between Cdc6p and Cdk1p was shown to have relevance to the later mechanism of control. At the G1/S transition, Cdc6p is abruptly degraded, and the targeted degradation of Cdc6p is thought to be a key mechanism by which the cell closes the window of opportunity for origin priming. By extensive mutagenesis, phosphorylation of Cdc6p at two clusters of sites was shown to be required for this cell cycle dependent degradation. Degradation is furthermore mediated by ubiquitination, as was demonstrated by in vitro reactions. Genetic experiments suggest that stabilizing Cdc6p may lead to unscheduled initiation of replication.

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Chapter 1: The Initiation of DNA Replication in *Saccharomyces cerevisiae:* An Introduction

The General Model for the Replication of Chromosomes

In 1963, Jacob, Brenner and Cuzin proposed a general model for the initiation of DNA replication. They proposed that the initiation of DNA replication was mediated by a cis-acting element on the chromosome, called a replicator, where new rounds of DNA replication would begin. Second, they proposed the existence of an initiator that would act in trans to promote DNA replication from the replicator. There are numerous examples of such replicators, or origins of replication, which have been identified for bacteria, bacteriophages, and mammalian viruses. In many of these cases, proteins that physically bind to the origin in a sequence specific manner have been identified. Origin recognition proteins, however, are not sufficient to mediate initiation. Other factors are typically required to convert origins bound by recognition proteins from a closed region of double stranded DNA to an open complex which supports the assembly of the DNA polymerases and accessory factors which will replicate the DNA.

Licensing of DNA replication: The Rao and Johnson Experiment

An early indication that cells are competent to replicate only at certain points in the cell cycle came from experiments published in 1970 by Rao and Johnson. In these experiments, cells from different points in the cell cycle were fused and DNA replication in each nucleus was evaluated. When S phase cells were fused with G1 cells, DNA replication in the G1 nucleus was induced, which suggested that S phase cells contain a signal that promotes DNA replication. However, fusion of S phase cells with G2 cells did not promote initiation in G2 nuclei, which indicated that only G1 nuclei were competent to

respond to the initiation signal present in S phase cells. This S phase promoting signal appeared to be absent in G2 cells, as fusion of G1 cells with G2 cells did not advance the timing of initiation in the G1 nuclei. Taken together, these experiments indicated that G1 nuclei are licensed for replication, and after one round of synthesis this license is revoked until the completion of mitosis. The current molecular interpretation of the Rao and Johnson experiments is that the proteins that license the genome are conceptually similar to the initiators described by Jacob, Brenner, and Cuzin. As will be apparent from the discussion that follows, the licensing proteins include not only the origin recognition proteins but also the whole gamut of proteins that assemble at origins in a cell cycle specific manner. The signal that triggers firing at these primed origins in *Saccharomyces cerevisiae* is generally thought to be mediated by Clb/Cdk1p and Dbf4p/Cdc7p, collectively referred to as S phase promoting factor.

Maintaining Genomic Integrity

Upon cell division daughter cells must emerge with a complete copy of the genome in order to be viable. In *E. coli*, in which the circular genome is replicated from a single origin sequence, more than one round of DNA replication typically begins within a given cell cycle. This secondary initiation occurs at a regular interval after the first initiation, and reinitiation depends on the eventual methylation of origin DNA. Importantly, the integrity of the *E. coli* genome is not compromised by this circumstance because one full copy of the genome is segregated into each daughter cell, even when a second round of synthesis is underway. However, the constraints on DNA replication in higher organisms are much greater. The genomes consist of multiple linear chromosomes, each of which are replicated from a multitude of origins. In order to duplicate the genome exactly in the course of S phase, each origin must fire no more than once. Unregulated origin firing would result in a web of DNA which could not possibly be segregated into two daughter cells. In practice, it

has been shown that even small perturbations in the chromosomal DNA are sufficient to cause cell cycle arrest before mitosis, including single strand breaks, double strand breaks, and even the assembly of licensing complexes. In addition, fusion of G1 or S phase cells with G2 cells in the Rao and Johnson experiment caused a delay in mitosis in the G2 nuclei, providing another indication that cells monitor the course of DNA replication, and that mitosis will not proceed until replication is complete.

Tools of the Trade

The techniques used for the analysis of DNA replication in *S. cerevisiae* are described here, particularly because the use of new techniques in the past few years has greatly contributed to our understanding of the initiation of DNA replication. In addition, discussion of techniques is generally warranted because the techniques used shape the models that evolve from the research. Molecular analysis of the cell cycle has been greatly accelerated by the existence of conditional-lethal *S. cerevisiae* mutants. These mutants have allowed the identification of many DNA replication genes, and also allow a convenient means of synchronizing cells at various stages of the cell cycle. Cells can also be synchronized by the addition of alpha factor, which causes the cells of the appropriate mating type to exit from the mitotic cell cycle in G1; hydroxyurea, which halts DNA synthesis due to the inhibition of ribonucleotide reductase and presumably depletion of the deoxynucleotide triphosphates; and nocodazole, which blocks mitosis by inhibiting the formation of the mitotic spindle. Fluorescence microscopy can be used to identify the nucleus, mitotic structures, bud scars, and the subcellular localization of proteins of interest, although this is somewhat limited by the small size of yeast. While light microscopy cannot be used to determine the DNA content of yeast cells per se, cell cycle mutants were first identified based on a homogenous morphology of cells arrested at the

nonpermissive temperature, and these morphologies occasionally suggest interpretations that can then be followed up with more sensitive techniques.

In the analysis of DNA replication in vivo, there are a number of techniques in use that provide information about the state of chromosomal DNA. Flow cytometry is used to evaluate the cell cycle stage or stages of a population of cells by generating a profile of the DNA content of that population. This technique cannot reliably identify cells with almost completely replicated or almost completely unreplicated DNA, but the integrity of chromosomes can be evaluated by pulse field gel analysis, as completely replicated or completely unreplicated DNA will migrate into the gel and partially replicated DNA will fail to do so. Semiconservative replication is still evaluated by density transfer, the technique originally used by Meselsohn and Stahl in 1957 to demonstrate the phenomenon. Origin firing itself is monitored by the use of two-dimensional (2D) agarose gels, which takes advantage of the fact that restriction fragments composed of linear DNA, DNA into which one fork has migrated, and DNA with a replication bubble all migrate differently on these gels.

A variety of techniques have also been used to evaluate the presence of proteins associated with yeast chromatin. Since yeast origins are known, a picture of the changing state of chromatin at the origin has been generated by the use of in vivo footprining. The association of specific proteins with origins in vivo can be monitored by the chromatin immunoprecipitation (CHIP) assay, which relies on crosslinking proteins to DNA with formaldehyde, shearing the chromatin, immunoprecipitating proteins of interest that are still chromatin bound, and amplifying the immunoprecipitated DNA with primers to sequences of interest. This technique has been used to demonstrate the association of specific proteins with yeast origins, and has also been used to monitor the progression of the DNA replication fork. A simpler method for detecting the association of proteins with DNA is to

purify chromatin and blot the associated proteins, although this method does not yield information about the chromosomal context in which these proteins bind.

Origins of DNA Replication

Understanding the nature of replicators and initiators is crucial to understanding the mechanism and regulation of the initiation of DNA replication. The *Saccharomyces cerevisiae* replicators were originally identified as chromosomal elements that would allow the episomal maintenance of plasmids, and are called autonomous replicating sequences (ARSs) (reviewed in Campbell and Newlon, 1991; Fangman and Brewer, 1991; Newlon and Theis, 1993; and Rowley *et al.*, 1994). A subset of these ARSs have been shown to act as origins in vivo by the use of 2D agarose gels. Using synchronized cells, it has been determined that while most origins fire close to the G1/S transition, some fire much later in S phase (Reynolds *et al.*, 1989). Interestingly, the late-firing origins tend to be proximal to telomeres. In addition, some late origins have been shown to maintain their late activation time when present on plasmids (Friedman *et al.*, 1996).

Deletion analysis of *ARS1* defined small elements required for ARS function, the most critical of which is called domain A (Celniker *et al.*, 1984). Analysis of functional ARSs has shown that domain A, or the ARS consensus sequence (ACS) is conserved. In addition to functioning as the critical element in yeast origins, the ACS has also been shown to be involved in the transcriptional repression of the silent mating type loci. The identification of the yeast origins provided an essential means for both the identification of the probable initiator protein in *S. cerevisiae*, ORC, as well as a means of studying the assembly of initiation proteins at this site in vivo.

The License to Replicate

In vivo footprinting of yeast origins has shown that origins that are properly licensed for replication show a distinctly different footprint as compared with origins that are not licensed (Cocker *et al.*, 1996). Over the course of the cell cycle, chromatin in the vicinity of the yeast origin exists in two states (Diffley *et al.*, 1994). The origin footprint from the G1/S transition to late mitosis is thought to be generated by the association of the origin recognition complex (see below) and Abf1p, a transcription factor required for the initiation of DNA replication (Rhode *et al.*, 1992, Appendix of this thesis). During the exit from mitosis, the origin footprint expands and persists in this altered state due to the presence of additional initiation factors that associate with the origins. This complex is referred to as the prereplicative complex and is thought to constitute a licensed origin. At the G1/S transition, upon activation of the S phase cyclin-dependent kinases (cdks), origins fire, and the footprint at the origin is converted from the prereplicative to the postreplicative complex. The proteins required for the formation of the preRC are described below.

ORC

The origin recognition complex (ORC) was purified by assaying for proteins that would footprint the ACS (Bell and Stillman, 1992) and the adjacent B1 element. ORC fails to bind nonfunctional copies of the ACS, indicating that the association of ORC with ARSs is limited to functional ARSs. The six subunits of ORC were identified by reverse genetics and found to be essential genes. Two subunits of ORC, Orc2p and Orc5p, were also identified by complementation of mutants that would allow derepression of a synthetic silencer the function of which depended on the ACS. A role for all of the ORC subunits in DNA replication has been demonstrated, either by showing by flow cytometry that mutants arrest with a 1N DNA content, or that mutants are defective in origin firing, as evaluated by 2D gel analysis. Most interestingly, in vivo footprints of the ACS and B1 elements in asynchronous cells have shown that the footprint obtained in vivo closely matches the

footprint generated by purified ORC in vitro, giving strong evidence that ORC associates with yeast origins in vivo (Diffley and Cocker, 1992).

Sequence analysis of the ORC subunits revealed that Orc1p and Orc5p each contain the Walker A and B motifs characteristic of ATP binding proteins (Walker *et al.*, 1982, Koonin, 1993), and mutational analysis of these motifs has shown that the ATP binding or ATP hydrolysis function of these subunits is critical for the essential function of ORC in vivo (Klemm *et al.*, 1997). In addition, these subunits have been found to directly interact with ATP. The bases within origins contacted by ORC have been extensively mapped by modification interference, in which specific bases are modified with a bulky adduct, and by missing contact analysis, in which a base is removed from the origin DNA (Lee and Bell, 1997). This analysis, coupled with crosslinking analysis, has shown that Orc1p, Orc2p, Orc3p, and Orc4p all directly contact the ACS in two different ARSs, that Orc5p contacts the B1 element, and that Orc6p both contacts the ARS outside of the regions essential for function and is not strictly required for ACS footprinting in vitro. Thus, through careful in vitro analysis, various roles for the ORC subunits in origin recognition are beginning to emerge.

Cdc6p

The *cdc6-1* mutant was one of the original cell cycle mutants found in the Hartwell screen, and was described as defective in DNA replication although mutant cells were found to arrest with 2N DNA (Culotti and Hartwell, 1971; Hartwell *et al.*, 1973; Hartwell, 1973). The *CDC6* gene was cloned by standard complementation (Zhou *et al.*, 1989), and was also identified in a screen for genes that would rescue a conditional mutant of *Schizosaccharomyces pombe* that suffered from premature passage into mitosis (Bueno and Russell, 1992). *cdc6-1* mutants were eventually shown to arrest with unreplicated DNA (Bueno and Russell, 1992), and Cdc6p was implicated specifically in the initiation of DNA

replication by use of a differential plasmid maintenance assay (Hogan and Koshland, 1992). In this assay, cell cycle mutants were evaluated for defects in initiation of DNA replication by comparing the plasmid loss rate for plasmids containing either one ARS sequence or seven tandem ARS sequences. Mutants in which plasmid loss was suppressed by the addition of multiple origins were construed as defective in the initiation of DNA replication. For *cdc6-1*, the rate of plasmid loss was reduced six-fold by the presence of multiple origins. Cdc6p was found to be required for both establishing and maintaining the preRC (Cocker *et al.*, 1996), which confirmed that Cdc6p plays a key role in initiation. Cdc6p was also shown directly to be present at yeast origins in G1 by the use of CHIP assays (Tanaka *et al.*, 1997), and this association is deficient in *orc1* mutants, suggesting that Cdc6p only assembles onto origins bound by ORC. Consistent with this, Cdc6p has been shown to associate in vitro with ORC (Liang *et al.*, 1995).

Analysis of the *CDC6* sequence revealed the presence of Walker A and Walker B motifs. Mutation of the consensus lysine in the Walker A motif to glutamate renders the protein non-functional in vivo (Perkins and Diffley, 1998; Jong, Elsasser and Campbell, unpublished), and the mutant protein specifically fails to support formation of the prereplicative complex (Perkins and Diffley, 1998). An allele of *CDC6* carrying a mutation in the Walker B motif, *CDC6-d1*, was found in a screen for dominant negative mutants of *CDC6* (Perkins and Diffley, 1998), and while the Cdc6-d1 protein supports the formation of the prereplicative complex, the Mcms (see below) fail to assemble onto chromatin. *CDC6* also bears homology to the central region of *ORC1*, and aside from the fact that the Walker A and B motifs of each of these proteins are required for in vivo function, the relevance of the homology is not understood.

Analysis of the *CDC6* transcript revealed that the gene is expressed periodically in the cell cycle (Zhou and Jong, 1990), and there are two waves of expression: one in late mitosis and one in late G1 (Zhou and Jong, 1990; Piatti *et al.*, 1995). Many DNA

replication genes are transcribed periodically in the cell cycle, but unlike most DNA replication genes, Cdc6p has been demonstrated to be an unstable protein (Piatti *et al.*, 1995; Drury *et al.*, 1997; Elsasser *et al.*, 1998). Degradation of Cdc6p may therefore be a mechanism by which the cell triggers initiation and limits initiation to once per cell cycle. Constitutive expression of Cdc6p has been shown to delay cells in G2 (Bueno and Russell, 1992) and impair cell growth in some strain backgrounds (Elsasser *et al.*, 1996; Elsasser *et al.*, 1998), suggesting that high levels of Cdc6p in G2 may activate a checkpoint that impairs mitosis, which in turn implies that degradation of Cdc6p is required for the timely advance of the cell cycle.

The Mcm Family

The Mcm family is comprised of six homologous proteins, currently referred to as Mcm2p, Mcm3p, Mcm4p (Cdc54p), Mcm5p (Cdc46p), Mcm6p, and Mcm7p (Cdc47p) (reviewed in Dutta and Bell, 1997). The original members of this family, Mcm2p and Mcm3p, were found in a screen for mutants with an enhanced plasmid loss rate (Maine *et al.*, 1984). Mcm4p (Cdc54p) and Cdc45p (described below) were found by analyzing existing collections of cold sensitive mutants for those with defects in DNA replication (Moir *et al.*, 1982), and Mcm5p (Cdc46p) and Mcm7p (Cdc47p) were found as temperature-sensitive mutants that could suppress the cold-sensitive *cdc45* and *cdc54* alleles (Moir *et al.*, 1982). Mcm6p was identified as a result of the yeast genome sequencing project. The Mcms each possess a putative ATP binding site, and mutations at these sites knock out function in vivo (reviewed in Dutta and Bell, 1997). Two-hybrid analysis shows that the members of this family physically interact with each other (Lei *et al.*, 1996, Dalton and Hopwood, 1997), and also with Dbf4p/Cdc7p (Lei *et al.*, 1997), a component of the S-phase promoting factor. In vitro analysis of purified murine Mcm complexes has shown that a complex of Mcm4p, Mcm6p, and Mcm7p possesses DNA

helicase activity (Ishimi, 1997), and the helicase activity is inhibited by the presence of Mcm2p (Ishimi *et al.*, 1998). Mcm4p and Mcm7p have been shown to associate with origin DNA using the CHIP assay (Aparicio *et al.*, 1997, Tanaka *et al.*, 1997), and this interaction fails in *orc1* and *cdc6* mutants held at the nonpermissive temperature, suggesting that the loading of the Mcms at yeast origins depends upon the prior association of ORC and Cdc6p. The levels of the Mcms appear to be constant throughout the cell cycle, but the subcellular localization and phosphorylation state varies (see below).

Cdc45p

Studies of Cdc45p suggest that it acts in concert with ORC and the Mcm proteins to drive the initiation of DNA replication. *cdc45* mutants are defective for entry into S phase (Hennessy *et al.*, 1991; Zou *et al.*, 1997), and show genetic interactions with five of the six Mcms, as well as *ORC2* and *ORC5* (Moir *et al.*, 1982; Hennessy *et al.*, 1991; Hopwood and Dalton, 1996; Zou *et al.*, 1997). In addition, Cdc45p has been shown to interact with Mcm7p/Cdc47p in a two hybrid assay, and to fractionate with Mcm5p/Cdc46p and Mcm7p/Cdc47p on gel filtration columns (Dalton and Hopwood, 1997). Plasmids with multiple ARSs are much more easily maintained in *cdc45* mutants than plasmids bearing a single origin, similar to the phenomenon observed in *cdc6* cells and suggestive of a defect in initiation (Hardy, 1997). Cdc45p has been shown to associate with yeast origins using the CHIP assay, suggesting that Cdc45p is a component of the prereplicative complex (Aparicio *et al.*, 1997). *CDC45* is not homologous to the Mcms, although it appears to function as a subunit of the Mcm complex. Reciprocal shift experiments with *cdc45*^{cs} *cdc7*^{ts} and *cdc45*^{c} *dbf4*^{ts} double mutants show that Cdc45p and Dbf4p/Cdc7p function interdependently in initiation (Owens *et al.*, 1997).

S Phase Promoting Factor

The firing of origins necessarily includes the transition from the closed, double stranded DNA at the yeast origin to the formation of an open complex which allows the assembly of the polymerases and accessory factors that synthesize DNA. Only origins that are licensed, or primed for initiation will fire, and the firing of these origins requires activation by an activity described as the S phase promoting factor. In yeast, the priming of origins requires, at the very least, the assembly of ORC, Cdc6p, the Mcm family, and Cdc45p at origins. The firing of yeast origins has been shown to require two kinases: Clb/Cdk1p and Dbf4p/Cdc7p.

Clb5,6p/Cdk1p

Cell cycle transitions in yeast correspond to, and are thought to be caused by, waves of cyclins that appear in the cell cycle and activate the cyclin dependent kinase Cdc28p, also called Cdk1p. Entry into mitosis is governed by the B-type cyclins Clb1p and Clb2p, and the exit from mitosis depends on the degradation of these cyclins. After mitosis, the Clns, including Cln1p, Cln2p and Cln3p, appear and are present throughout G1. During early G1, cells can respond to mating pheromone, which in turn causes cells to exit from the cell cycle, mate, and then return to the cell cycle. Exit from the cell cycle depends on the inhibition of the Cln1p and Cln2p by Far1p. Midway through G1, cells will no longer respond to mating pheromone, and this point cells are committed to another cell cycle. The first wave of B-type cyclins or Clbs that appears in the cell cycle, comprised of Clb5p and Clb6p, is thought to govern the G1/S transition.

An early indication of a requirement for Clb5p and Clb6p in DNA replication came from the observation that cells deleted for both *CLB5* and *CLB6* showed substantial delay in S phase (Schwob and Nasmyth, 1993). Clb5p and Clb6p appear well in advance of the G1/S transition and associate with Cdk1p, but Clb5p/Cdk1p and Clb6p/Cdk1p are

maintained in an inactive state by the presence of the inhibitor Sic1p (Schwob *et al.*, 1994). Degradation of Sic1p by the Cdc4 pathway and subsequent activation of Clb5,6p/Cdk1p has been demonstrated to be the primary control point for the initiation of S phase by several lines of evidence. First, *cdc4* mutants ordinarily arrest with unreplicated DNA, but deletion of *SIC1* in a *cdc4* mutant resulted in progression of cells through S phase, with a subsequent arrest in G2 (Schwob *et al.*, 1994). Second, the onset of DNA replication is premature in a *ΔSIC1* strain and correlates with the appearance of Clb5p and Clb6p (Schwob *et al.*, 1994). Third, the expression of a stabilized allele of Sic1p causes delayed initiation of DNA replication (Verma *et al.*, 1997). Thus, by all in vivo measures, initiation of DNA replication is triggered by the activation of Clb/Cdk1p. In spite of this fact, the substrates that are phosphorylated in an event that causes initiation are completely unknown.

Dbf4p/Cdc7p

Like *cdc6-1*, the *cdc7-1* mutant was recovered in the Hartwell screen for cell cycle mutants (Hartwell *et al.*, 1973), and genetic characterization indicated that Cdc7p is likely to function late in the initiation process (Hartwell, 1978). Biochemical characterization showed that Cdc7p possesses histone H1 kinase activity (Yoon and Campbell, 1991; Hollingsworth and Sclafani, 1990). The activity of Cdc7p varies during the cell cycle, and peaks at the G1/S transition (Yoon *et al.*, 1993; Jackson *et al.*, 1993). Notably, Cdc7p kinase activity is active at the *cdc4* block, indicating that the kinase activity is not activated by Clb/Cdk1p, although Cdc7p may be activated by Cln/Cdk1p, as overexpression of *CDC7* partially suppresses an allele of *cdc28* that arrests in G1 at the nonpermissive temperature (Yoon *et al.*, 1993). Interestingly, analysis of origins in a *cdc7* mutant has shown that the prereplicative complex is maintained at origins at the nonpermissive temperature (Diffley *et al.*, 1994), in spite of the fact that Clb/Cdk1p activity is high (Amon

et al., 1992). Prereplicative complexes at origins in the presence of high Clb/Cdk1p activity is rare, and the fact that this does occur in a *cdc7* mutant is perhaps the most direct piece of evidence indicating a role for Cdc7p in origin firing. Cdc7p function is not required after the hydroxyurea step, although reciprocal shift experiments show that the completion of S phase is substantially slowed in *cdc7* mutants incubated in hydroxyurea and then shifted to the nonpermissive temperature (Hereford and Hartwell, 1974; Hartwell, 1974). Recent data has shown that Cdc7p is not only required for the firing of early origins, but also for the firing of both early origins and late origins (Bousset and Diffley, 1998; Donaldson *et al.*, 1998).

Cdc7p, however, does not act alone in origin firing. The genetic evidence suggesting that Dbf4p is the activating subunit of Cdc7p includes observations that high copy *CDC7* will suppress *dbf4* mutants and vice versa, and that *dbf4* and *cdc7* are synthetically lethal (Kitada *et al.*, 1993). Cdc7p and Dbf4p have been shown to interact in vivo by two hybrid analysis (Jackson *et al.*, 1993) and in vitro (Dixon and Campbell, 1997). Moreover, Dbf4p is required for high activity of Cdc7p (Dixon and Campbell, 1997). Consistent with a role in DNA replication, Dbf4p was found in a one hybrid screen for replication proteins, in which a strain bearing the ARS consensus sequence placed upstream of a reporter gene was transformed with a standard library of activation domain plasmids (Dowell *et al.*, 1994). Deletion analysis showed that the domains of Dbf4p required for origin binding and Cdc7p binding are separable, but both are required for the essential function of Dbf4p.

A screen for mutants that would bypass the *cdc7* DNA replication block recovered a mutant that would not only suppress both *cdc7* and *dbf4*, but would bypass a deletion of both genes (Jackson *et al.*, 1993). This mutant, *bob1*, was identified as an allele of Mcm5p (Hardy *et al.*, 1997), and this strongly suggested that the Mcms are substrates of

Dbf4p/Cdc7p. In a complementary screen, an allele of *DBF4* was identified as a suppressor of *mcm2-1*, and Mcm2p and Dbf4p were found to interact in the two hybrid system (Lei *et al.*, 1997). Individual Mcm proteins purified from yeast have been tested as substrates Dbf4p/Cdc7p produced in insect cells. Mcm2p and Mcm6p were found to be strongly phosphorylated, Mcm3p was moderately phosphorylated, Mcm4p and the mutant protein Mcm2-1p were phosphorylated poorly, and Mcm5p and Mcm7p were not phosphorylated at all (Lei *et al.*, 1997). Observation that the Mcms are substrates of Dbf4p/Cdc7p does not rule out that they are also substrates of Clb/Cdk1p, and in fact Xenopus Mcm4p has been shown to be phosphorylated by cyclinB/cdc2 (Hendrickson *et al.*, 1996).

Cell Cycle Dependence of Initiation

Control of origin licensing by Clb/Cdk1p

In vivo footprinting analysis of yeast origins has shown that the proteins associated with yeast origins oscillate between two states. In vivo footprints of the yeast origin from the G1/S transition to late mitosis correspond to the footprints generated in vitro by the association of ORC and Abf1p, and in late mitosis, the footprint expands due to the assembly of other factors, and remains this way until the G1/S transition. This thumbnail description of origin licensing derives from in vivo footprinting analysis of cells blocked with various cell cycle inhibitors, including alpha factor, hydroxyurea, and nocodazole, and by using strains defective in cell cycle progression, including *cdc28, cdc4, cdc7, cdc6, cdc17, cdc23, cdc16* and *cdc15* (Diffley *et al.*, 1994; Cocker *et al.*, 1996; Bousset and Diffley, 1998). The presence of the prereplicative footprint in cells arrested with alpha factor or at the *cdc28* block indicated that the prereplicative complex is already formed in early G1. Two observations argue that both components of S phase promoting factor, Clb/Cdk1p and Dbf4p/Cdc7p, are required for initiation: first, the prereplicative complex is

present at the *cdc4* arrest, in which Dbf4p/Cdc7p is active but Clb5p/Cdk1p is still inhibited by Sic1p, and second, the prereplicative complex is also present at the *cdc7* block, at which point Clb/Cdk1p is active. Conversion of the prereplicative complex to the postreplicative complex occurs early in S phase, as postreplicative complexes are found in cells arrested with hydroxyurea or at the *cdc17* (DNA polymerase α) block, both of which cause cells to arrest with nearly 1N DNA. Origins remain bound by the postreplicative complex when arrested at the *cdc16* or *cdc23* block in G2, or at the *cdc15* block in late mitosis. The observation that in *cdc6* cells arrested at the nonpermissive temperature with postreplicative origins was originally interpreted to mean that Cdc6p was not required for origin licensing (Diffley *et al.*, 1994). However, later experiments showed that in the absence of Cdc6p the prereplicative complex fails to form after release from a mitotic block, and that prereplicative complexes formed in *cdc6* cells at the permissive temperature could be disrupted by a shift to the nonpermissive temperature (Cocker *et al.*, 1996).

These in vivo footprinting studies revealed that the oscillations of the prereplicative and postreplicative complexes at replication origins correlated in general with the activation and inactivation of the Clb/cdks. Interestingly, a screen for mutants that would replicate DNA in G2 provided more evidence that Clb/cdks blocked the formation of the prereplicative complex (Dahmann *et al.*, 1995). Cells deleted for *CLB1, CLB3* and *CLB4,* temperature sensitive for *clb2* were mutagenized, synchronized in G2, and assayed for incorporation of bromodeoxyuridine at the nonpermissive temperature. Mutants which synthesized DNA under these conditions were shown to have decreased levels of Clb5p/Cdk1p, suggesting that this defect allowed for the licensing and firing of origins in G2. A role for the Clb/cdks in preventing licensing in G2 was shown more directly by synchronizing wildtype cells in G2 and driving *SIC1* from the *GAL* promoter. Under these conditions, Clb/Cdk1p activity was inhibited and the prereplicative complexes assembled at yeast origins. Subsequent suppression of *SIC1* transcription led to exactly

one more round of DNA replication, resulting in cells with 4N DNA. Thus, inactivation of the Clb/cdks in G2 is sufficient to promote DNA replication.

Window of opportunity for initiation

The observations that *CDC6* is transcribed periodically in the cell cycle and that the Cdc6p protein is unstable led to the hypothesis that the timely expression of *CDC6* is required for initiation of DNA replication (Piatti *et al.*, 1996). In experiments that have come to be referred to as "point of no return" experiments, *cdc15* cells with *CDC6* expression driven from the methionine-repressible *MET3* promoter were synchronized in late mitosis by shifting to the nonpermissive temperature. The expression of*CDC6* was repressed, cells were released from the mitotic block, and *CDC6* transcription was introduced at various points after the release from the block. When transcription of *CDC6* was introduced within 30 minutes after exit from mitosis, cells proceeded through a normal S phase. However, when the transcription of *CDC6* was delayed until 30 minutes or more after the release from mitosis, which coincided with the activation of Clb5p/Cdk1p, cells failed to replicate their DNA. A similar experiment was done using a *cdc15 MET-CDC6* strain deleted for the S phase cyclins *CLB5* and *CLB6*. In this strain, the window of opportunity for Cdc6p-dependent initiation was increased to 70 minutes, at which point the mitotic Clb/cdks became active. These observations demonstrated that in order to promote origin firing, Cdc6p must be expressed in the absence of the Clb/cdks, and that phosphorylation of Cdc6p by the Clb/Cdks was a likely mechanism by which cells prevent origins from firing more than once within a given cell cycle.

Assembly of Licensing Proteins at the Origin

As described above, chromatin immunoprecipitation assays have been used to show that ORC, Cdc6p, the Mcms, and Cdc45p all associate with yeast origins, and the current

thinking is that assembly of all of these components at the origin constitute origin licensing. These proteins specifically associate with origins in G1, and aside from ORC, are ordinarily not found at yeast origins in G2. The likelihood of sequential assembly of these licensing proteins at yeast origins was indicated by elegant experiments done in the Xenopus system, in which the assembly of both Cdc6p and the Mcms onto chromatin depended on the prior assembly of ORC, and that the assembly of the Mcms depended the presence of Cdc6p but not vice versa (Coleman *et al.*, 1996). By using temperature sensitive mutants in the licensing proteins and probing for the association of wild type licensing proteins at the origin, the same order of assembly has been observed in *S. cerevisiae*: the presence of ORC at origins allows the assembly of Cdc6p, and the Mcms and Cdc45p are subsequently loaded (Aparicio *et al.*, 1997; Tanaka *et al.*, 1997). Although Cdc6p is normally found to associate with yeast origins only in G1, ectopic expression of *CDC6* in G2 will drive the association of Cdc6p with origin DNA. Even in the presence of Cdc6p, however, the Mcms will not assemble at origins, suggesting that blocking this step in the licensing of origins is one mechanism by which cells limit DNA replication to one round per cell cycle. Indeed, in *cdc6-2* and *cdc6-3* cells, which moderate amounts of unscheduled initiation of DNA synthesis are observed, the Mcms are found to be associated with chromatin in both G1 and G2 (Liang and Stillman, 1997).

Degradation of initiation proteins

The prevalence of initiation proteins can in theory be controlled transcriptionally, translationally, or post-translationally. A variety of the DNA replication genes are expressed periodically in the cell cycle, peaking in G1 (reviewed in Koch and Nasmyth, 1994). Transcription of these genes in G1 is controlled by the SCB and MCB promoter elements, and not surprisingly, the G1 cyclin/cdks are required for SCB- and MCB-dependent transcription. In most cases, however, the levels of the encoded proteins do not

oscillate with the cell cycle as the transcripts do, except in the case of two initiation proteins: Cdc6p and Dbf4p. The levels of Cdc6p in the cell cycle mirror the presence of the transcript (Piatti *et al.*, 1995), not only because Cdc6p is an unstable protein, but also because Cdc6p is both expressed in and stable in G1. The observation that Cdc6p is rapidly degraded in S, G2 and M phases (Piatti *et al.*, 1995; Drury *et al.*, 1997), coupled with the observations that Cdc6p is phosphorylated by the Clb/cdks and not the Cln/cdks (Elsasser *et al.*, 1996) and that deletion of the Cdk1p interaction of Cdc6p with stabilizes Cdc6p (Drury *et al.*, 1997), suggests that degradation is dependent on phosphorylation by the Clb/cdks. Mutation of various consensus Cdk1 phosphorylation sites in fact results in the stabilization of Cdc6p (Elsasser *et al.*, 1998). Like Cdc6p, Dbf4p is stable in G1 and unstable in S phase and G2 (Yang and Campbell, unpublished observations), although whether the instability of Dbf4p correlates with its phosphorylation is unknown.

Cell cycle dependent proteolysis is emerging as a general mechanism by which cell cycle transitions are controlled (reviewed in King *et al.*, 1996; Deshaies, 1997). Targeted degradation of proteins is mediated by the covalent modification of these proteins with ubiquitin, which in turn depends upon the combined action of a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and in some cases on a ubiquitin protein ligase (E3). Degradation of Sic1p at the end of G1 depends on the Cdc34 pathway, named for the E2 involved (Schwob *et al.*, 1994; Verma *et al.*, 1997). Cdc6p also appears to be degraded by this pathway, as Cdc6p is stabilized in G2 in Cdc34 pathway mutants (Piatti *et al.*, 1996; Drury *et al.*, 1997), and in vitro ubiquitination of Cdc6p is dependent on Cdc34p (Elsasser *et al.*, 1998). Degradation of various proteins during the passage through mitosis, including the sister chromatid cohesion protein Pds1p and the mitotic cyclins, is mediated by the anaphase promoting complex, which appears to function as an E3. The dependence of the stability of Dbf4p on either of these pathways has not been evaluated.

Cell Compartmentalization of Initiation Proteins

Since DNA replication is the province of the nucleus, export of DNA replication proteins from the nucleus in a cell cycle dependent manner is one method by which initiation might be regulated. Localization studies with Cdc6p tagged with green fluorescent protein have shown that Cdc6p is present in detectable levels only in G1 cells and only in the nucleus (Luo, Elsasser, and Campbell, unpublished). Mutation of the nuclear localization signal causes stabilization of Cdc6p (Elsasser *et al.*, 1998), and while mutation of the Cdk1p consensus sites results in stabilization of Cdc6p, this mutant protein is still found in the nucleus (Elsasser *et al.*, 1998; Luo, Elsasser, and Campbell, unpublished). Thus, the localization of Cdc6p does not appear to be regulated by phosphorylation. The subcellular localization of the Mcms, however, does appear to be regulated, as the localization of the Mcm proteins is significantly altered as cells replicate their DNA. The Mcms are nuclear during G1, but are present primarily in the cytoplasm as cells enter S phase (Hennessy *et al.*, 1990; Yan *et al.*, 1993; Dalton and Whitbread, 1995). The mechanism by which the Mcms are redistributed has not been established, but targeted export rather than proteolysis coupled with new synthesis is likely, since the levels of the Mcms are constant throughout the cell cycle. In contrast, Cdc45p has been found to be nuclear throughout the cell cycle.

A Potential Role for Mitotic Proteins in DNA Replication

There are several proteins required for mitosis that have been implicated in DNA replication. These include Cdc5p, Cdc14p, and the anaphase promoting complex component Cdc16p. The *cdc5* mutant was recovered in the original Hartwell screen for cell cycle mutants, and cells incubated at the nonpermissive temperature arrest in late nuclear division (Hartwell *et al.*, 1973; Hartwell, 1973). Cloning of *CDC5* revealed that

Cdc5p is a member of the Polo family of kinases (reviewed in Hunter and Plowman, 1997), and a role for the Clb5p kinase in the destruction of mitotic cyclins is suggested because *cdc5* mutants arrest at the nonpermissive temperature with high Clb/cdk activity (Shirayama *et al.*, 1998). In addition, Cdc5p was shown to be an unstable protein, and expression of stabilized allele of *CDC5* causes cells to arrest in G2 with low levels of mitotic Cdk1p activity. The evidence implicating Cdc5p in DNA replication is based on the observations that *CDC5* was found to suppress *dbf4-1*, that Cdc5p phosphorylates Dbf4p, and that Cdc5p and Dbf4p were shown to interact by two hybrid analysis (Hardy and Pautz, 1996). Cdc5p does not interact with Cdc7p, suggesting that the association of the kinases with Dbf4p is mutually exclusive. The interaction of Dbf4p with Cdc5p depends on the N-terminal 80 amino acids of Dbf4p, a region that is dispensable for complementation of *dbf4-1* (Dowell *et al.*, 1994), and this in turn suggests that the interaction of Dbf4p with Cdc5p is not required for its essential function. The most significant piece of evidence that suggests a role for Cdc5p in initiation comes from the observation that the plasmid loss rate in *cdc5* mutants is suppressed by the presence of multiple origins on a plasmid.

Like *cdc5* mutants, *cdc14* mutants also arrest with an extended spindle and divided chromatin (Culotti and Hartwell, 1971, Hartwell *et al.*, 1973), and like *cdc5* mutants, *cdc14* mutants show a high plasmid loss rate that is suppressed by the presence of multiple origins on a plasmid (Hogan and Koshland, 1992). In addition, *cdc14* mutants held at the nonpermissive temperature arrest with high levels of mitotic cdk activity. Since Clb/Cdk1p has been shown to antagonize the licensing of origins, it is possible that the plasmid maintenance defects observed derive not from a direct role for Cdc5p and Cdc14p in initiation but are produced by prolonged activation of the mitotic kinases.

Like the *cdc5* and *cdc14* mutants, *cdc16* and mutants are defective in mitosis, but instead of arresting at telophase, *cdc16* mutants arrest with a short spindle and prior to

segregation of sister chromatids. In spite of the fact that Cdc16p is known to be required for degradation of the B-type cyclins and *cdc16* mutants arrested at the nonpermissive temperature show high levels of Clb/Cdk1p activity, *cdc16* mutants have been shown to aberrantly initiate DNA synthesis when held at the nonpermissive temperature (Heichman and Roberts, 1996). Although this result is controversial (Pichler *et al.*, 1997), this aberrant DNA synthesis has been shown to require Cdc6p and Mcm5p activity, begin at origins of replication, and initiate a second round of semiconservative DNA synthesis as judged by density transfer (Heichman and Roberts, 1998). Stabilization of Cdc6p could formally cause this result, but the stability of Cdc6p has been shown to be unaffected in APC mutants (Drury *et al.*, 1997). A role for Cdc16p, and by extension the anaphase promoting complex, in suppressing spurious initiation in G2 may be construed as Cdc16p also having a positive role in the initiation of DNA replication.

Summary

Yeast origins are licensed by the assembly of Cdc6p, the Mcms, and Cdc45p origins bound by ORC at the end of mitosis. At the G1/S transition, concomitant with the destruction of Sic1p and the activation of the S phase cyclin dependent kinases, origins of DNA replication fire. Origin firing is dependent not only on the Clb/cdks but also on the Dbf4p/Cdc7p kinase. The proteins that are phosphorylated by the Clb/cdks during the initiation of DNA replication are unknown, but a subset of the Mcm proteins are likely to be the crucial targets of Dbf4p/Cdc7p. After initiation of DNA replication, additional rounds of initiation are prevented by the continued activity of the Clb/cdks until the end of mitosis. In the presence of the Clb/cdks, Cdc6p is degraded, and the Mcms are exported to the cytoplasm. The observation that *cdc16* mutants replicate their DNA at the nonpermissive temperature suggests that the anaphase promoting complex has a role in blocking the relicensing of origins, possibly by degrading Dbf4p. After sister chromatids are segregated

at the end of mitosis, the mitotic cyclins are destroyed, Cdc6p is synthesized, the Mcms are imported, and origins are licensed for a new round of DNA replication.

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Chapter 2: Interaction Between Yeast Cdc6 Protein and B-type Cyclin/Cdc28 Kinases

(Suzanne Elsasser, Feng Lou, Bin Wang, Judith L. Campbell, and Ambrose Jong, *Molecular Biology of the Cell* 7, 1723-1735 (1996))

ABSTRACT

During purification of recombinant Cdc6 expressed in yeast, we found that Cdc6 interacts with the critical cell cycle, cyclin-dependent protein kinase Cdc28. Cdc6 and Cdc28 can be coimmunoprecipitated from extracts, Cdc6 is retained on the Cdc28-binding matrix p13-agarose, and Cdc28 is retained on an affinity column charged with bacterially produced Cdc6. Cdc6, which is a phosphoprotein in vivo, contains five Cdc28 consensus sites and is a substrate of the Cdc28 kinase in vitro. Cdc6 also inhibits Cdc28 histone H1 kinase activity. Strikingly, Cdc6 interacts preferentially with B-type cyclin/Cdc28 complexes and not Cln/Cdc28 in log phase cells. However, Cdc6 does not associate with Cdc28 when cells are blocked at the restrictive temperature in a *cdc34* mutant, a point in the cell cycle when the B-type cyclin/Cdc28 inhibitor $p40^{Sic1}$ accumulates, and purified $p40^{Sic1}$ inhibits the Cdc6/Cdc28 interaction. Deletion of the Cdc28 interaction domain from Cdc6 yields a protein that cannot support growth. However, when overproduced the mutant protein can support growth. Furthermore, whereas overproduction of wildtype Cdc6 leads to growth inhibition and bud hyperpolarization, overproduction of the mutant protein supports growth at normal rates with normal morphology. Thus, the interaction may have a role in the essential function of Cdc6 in initiation plus in restraining mitosis until replication is complete.

INTRODUCTION

The yeast Cdc6 protein appears to be essential for cell cycle specific activation of DNA replication origins. Early studies showed that *cdc6* mutants arrest at the nonpermissive temperature with 1N DNA and one large bud (Hartwell, 1976). Reciprocal shifts suggested a block in initiation, since *cdc6* mutants remain sensitive to hydroxyurea, which inhibits elongation, after shift down from the nonpermissive temperature and are insensitive to a temperature block after release from hydroxyurea arrest (Hartwell, 1976). Plasmids containing a single origin of DNA replication are unstable in *cdc6* mutants, but this plasmid loss phenotype is suppressed by the presence of multiple origins on a single plasmid, suggesting a direct interaction between Cdc6 and yeast ARSs (Hogan and Koshland, 1992). Furthermore, analysis of replication intermediates formed at the nonpermissive temperature in a *cdc6* mutant show that origin firing is defective (Liang *et al.*, 1995). The Cdc6 protein produced in baculovirus-infected insect cells has been shown to co-immunoprecipitate with yeast ORC, the six protein complex that binds specifically to ARSs and is required for the initiation of DNA replication (Bell *et al.*, 1993; Bell and Stillman, 1992; Liang *et al.*, 1995). In fact, Cdc6 is highly homologous to the largest subunit of ORC, *ORC1* (Bell *et al.*, 1995). Recent evidence suggests that Cdc6 associates with ORC late in mitosis or early in G1, remains associated with ORC during G1, but is not associated with ORC after origin firing for the duration of S phase and G2, and early M (Cocker *et al.*, 1996; Diffley and Cocker, 1992; Diffley *et al.*, 1994; Rowley *et al.*, 1995). Expression of the *CDC6* gene is periodic, mRNA levels accumulating transiently in late G1 and then again in late M or early G1 (Bueno and Russell, 1992; Piatti *et al.*, 1995; Zhou and Jong, 1990). Unlike other replication proteins, the Cdc6 protein appears to be unstable, and its intracellular levels mirror mRNA accumulation (Piatti *et al.*, 1995). If expression of Cdc6 is turned off in growing cells, they fail to initiate DNA replication upon depletion of Cdc6 but undergo an aberrant mitosis, segregating unreplicated

chromosomes (Piatti *et al.*, 1995). A homolog of *CDC6* , called cdc18^{+}, exists in *S. pombe* (Kelly *et al.*, 1993). *cdc18* mutants are defective in the initiation of DNA replication and furthermore, *cdc18* mutants, like *cdc6*, fail to arrest in mitosis, but divide with unreplicated chromosomes. In summary, Cdc6 has the properties of a prime regulator of replication initiation, but molecular evidence for its precise mechanism of action is very limited.

An unexplained property of Cdc6 is that *CDC6* rescues mitotic catastrophe in *S. pombe cdc2 wee1* mutants and overproduction under a *GAL* promoter leads to a G2 delay in *S. cerevisiae* (Bueno and Russell, 1992). This observation has been used to suggest that Cdc6 is part of a signal that restrains mitosis until S phase is complete. It has recently been shown that the G2 delay observed in *S. cerevisiae* upon overexpression of Cdc6 requires active Clb5,6 and that constitutive overproduction of Clb5 leads to an identical G2 delay as overproduction of Cdc6 (Basco *et al.*, 1995). Clb5,6 is required for entry into S phase and interestingly, also perhaps for preventing re-replication in the G2 phase of the cell cycle (Dahmann *et al.*, 1995; Epstein and Cross, 1992; Kuhne and Linder, 1993; Richardson *et al.*, 1992; Schwob and Nasmyth, 1993). Overproduction of *CLB5* also leads to constitutive overproduction of Cdc6 mRNA, which might account for the similar phenotypes. However, the phenotype also might reflect a direct interaction. Either explanation is consistent with the observation that *cdc6*^{ts} mutants are suppressed by overproduction of *CLB5* (Basco *et al.*, 1995).

We have recently found that Cdc6 interacts with Cdc28, is specific for B-cyclin/Cdc28 complexes but not Cln/Cdc28 complexes and that the association is cell cycle regulated. Deletion of the Cdc28 interaction domain has multiple effects on the cell cycle. We propose that this association is important for the regulation resulting in the correct ordering of DNA replication and mitosis in the cell cycle.

MATERIALS AND METHODS

Strains

GST and fusion proteins GST-Cdc6 and GST-Cdc6/K114E were expressed in **BJ2168** (*a prc1-407 prb1-1122 pep4-3 leu2 trp1 ura 3-52*) and **RJD635** (*a ura3 leu2 trp1 pep4::TRP1 cdc28::CDC28-HA::TRP1 bar1::LEU2*), which contains hemagglutinin-tagged Cdc28. GST and GST-Cdc6 were also expressed in strain **RJD561** (*a cdc28-4 ura3 leu2 pep4::TRP1*).

Tagged cyclins for kinase and inhibition assays were prepared from **YMT263** (*a cln2::CLN2-HA-LEU2 ade2-1 trp1-1 leu2-3, 112 ura3 GAL+*), **K3819** (*a ura3::ADH-HA-CLB5-URA ade2-1 trp-1 leu2-3,112 GAL*$^+$) , and **YMG17** (*a leu2-3, 112::GAL-CLB2-HA-LEU2 ade2-1 trp1-1 ura3 GAL*$^+$) (Peter and Herskowitz, 1994).

Extracts containing tagged cyclins for binding experiments were prepared from **RJD934** (*a can1-100, leu2-3,112, his3-11,15, trp1-1, ura3-1, ade2-1, pep4::TRP1, leu2::GAL-CLN2-HA::LEU2::URA3*), **K3819**, and **JCY61**. Strain **JCY61** was prepared by crossing **K3819** with **RJD668** (*a leu2, trp1, ura3, pep::TRP1, cdc34-2*) sporulating, and selecting for segregants which were prototrophic for uracil and temperature sensitive for growth. (All RJD strains were the gift of Ray Deshaies, Caltech, Pasadena, CA).

Complementation experiments were carried out with **K4055** (a *leu2-3, can1-100, ade2-1, GAL, CDC6::hisGURA3hisG, trp1-1::TRP1 MET-CDC6*), with *URA3* looped out (Piatti *et al.*, 1995), in which the only copy of *CDC6* is controlled by the methionine repressible *MET3* promoter. Incubation of this strain in 2mM methionine yields a conditional null.

Plasmids

Plasmids for the expression of GST fusion proteins in yeast were prepared as follows: YGp10, a yeast vector containing the *GAL1,10* promoter, was prepared by cloning an EcoRI fragment containing the entire *GAL1,10* promoter into the EcoRI site of YEp352 Hill *et al.*, 1986 with the BamHI site in the promoter cassette proximal to the polylinker. YGpKTU6, encoding GST-Cdc6, was generated in two steps. *CDC6* was first amplified from the PstI site to the end of the gene (Zhou *et al.*, 1989), and the resulting PstI-HindIII fragment was cloned into the same sites in YGp10, generating YGp10-PH6. The N terminus of the fusion protein was created by amplifying the bacterial GST fusion protein (described below) from the start codon through the PstI site. The resulting SacI-PstI fragment was cloned into the same sites of YGp10-PH6, resulting in YGpKTU6. YGpME6, encoding GST-Cdc6/K114E, was generated by replacing a PstI fragment of YGpKTU6 containing both gene and vector sequence, with a similar fragment in which lysine 114 had been mutated to glutamate. pRD56a contained GST alone driven by the *GAL1,10* promoter, and was provided by Ray Deshaies.

Plasmids for the expression of *CDC6* and *cdc6ΔN*, a mutant missing the N terminal 47 amino acids, from the *GAL1,10* promoter were generated as follows: YGp618, containing the *CDC6* gene, was generated by cloning *CDC6* from the SspI site upstream of the translational start site to the SpeI site downstream of the stop codon into YGp10. pSE19, containing *cdc6ΔN*, was generated by cutting YGp618 with BamHI and PstI, which cuts *CDC6* at residue 48, and replacing the fragment with an oligonucleotide cassette comprised of SE27 and SE28, allowing for expression from residue 48 of *CDC6*, *cdc6ΔN*.

Plasmids for the expression of *CDC6* and *cdc6ΔN* from the natural promoter were prepared as follows: The *CDC6* promoter from the XhoI site to the translation start site was amplified by PCR using oligos SE23 and SE24. SacI and BamHI sites were introduced into the 5' and 3' ends respectively. This promoter was cloned into pBluescript

SK(-), generating pSE14. The full length *CDC6* open reading frame was amplified by PCR using oligonucleotides SE35 and SE37, which introduced BamHI and NdeI sites upstream of the coding sequence, and a HindIII site downstream. This fragment was cloned into the BamHI and HindIII sites of pSE14 to generate pSE20. For expression in yeast, pSE20 was cut with SacI and HindIII, and the resulting fragment containing both promoter and coding region were cloned into the same sites in pRS315 (Sikorski and Hieter, 1989) to generate pSE26. *cdc6ΔN* was amplified by PCR using oligos SE36 and SE37, which introduced BamHI and NdeI sites just upstream of the PstI site and a HindIII site downstream of the stop codon. This fragment was cloned into the BamHI and HindIII sites of pSE14 to generate pSE21. pSE21 was cut with SacI and HindIII and cloned into pRS315 to generate pSE27.

Oligonucleotides

SE23 GCGAAACCTAGAGGCTTTCTCGAGGCAAAAATTTTTCCAA

SE24 TCAATAGTACCATATACGTTCATGTCAGCTATACCAA

SE27 GATCCGGCATATGGGCGGCCTGCA

SE28 GGCCGCCCATATGCCG

SE35 GGAGGATCCGGAGGCCATATGTCAGCTATACCAATAACT

SE36 GGAGGATCCGGAGGCCATATGCTGCAGTTTGGCTCAC

SE37 GGCCAAAGCTTCAATTGTACCCCACCTC

Expression and purification of GST and fusion proteins from yeast.

Proteins were expressed in the yeast strains BJ2168 and RJD635 as follows: Cells were grown to a density of $2x10^7$ cells/ml in minimal medium containing raffinose, induced with 2% galactose, and harvested after 4 h. Extracts were prepared by glass bead lysis in Buffer A (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% Nonidet P-40, 10%

glycerol, 1.5 mM DTT, 2 mM EDTA, 1 mM EGTA) with protease inhibitors (60 μg/ml AEBSF, 160 μg/ml benzamidine, 1μg/ml aprotinin, 1μg/ml leupeptin and 1 μg/ml antipain), and cleared by spinning at 35,000 rpm for 20 minutes at 4°C. Extract was incubated with glutathione agarose beads at a ratio of 200 mg of extract per ml of matrix for three h at 4°C with gentle agitation. Beads were washed extensively with Buffer A, then with Buffer B (Buffer A with 0.15 M salt in place of 0.5 M salt). Protein was eluted with 5 mM reduced glutathione in Buffer B by incubating overnight at 4°C. Expression from RDJ561 was as described above, except that cells were grown at 23°C.

Expression and Purification of bGST-Cdc6 Proteins and Charging of Beads with the Recombinant Proteins

All vectors for bacterial expression were based on pGEX-2T. bGST-CDC6 contains GST fused to the *CDC6* open reading frame. GST-NB contains GST fused to *CDC6* from the first ATG to the BglII site at residue 190. GST-NP contains GST fused to *CDC6* from the first ATG to the PstI site at residue 48. GST-BE contains GST fused to a C-terminal fragment beginning at the BglII site at residue 190 and ending at the natural termination codon. Proteins were expressed by growing *E. coli* DH5a cells containing these vectors to an OD_{600} of 0.6, inducing with 1mM IPTG and shifting to 30°C, and harvesting after 4 h. Extracts were prepared by lysing cells in Buffer C (50 mM Tris-HCl, pH 7.2, 50 mM NaCl, 10 mM DTT, 10% glycerol, 0.5% NP-40, 0.5 mM EDTA) with protease inhibitors (as above). For each fusion protein, beads were prepared by incubating extract (3-5 mg of protein) of cells expressing fusion protein with 60 μl of glutathione agarose beads for 1 h, then washing in lysis buffer.

Preparation of Hemagglutinin Epitope-tagged Cyclins

For preparation of extract for either kinase or binding assays, strains were grown as follows: RJD635, YMT263 and K3819 were grown in YPD to a density of $4x10^7$ cells/ml and harvested. YMG17 cells were grown in YPRaffinose, expression of HA-Clb2 was induced with 2% galactose at a density of $2x10^7$ cells/ml and harvested after 2 h. RJD934 cells were grown to saturation in YPD, inoculated into YPGalactose at a dilution of 1:500, grown for 16 h and harvested. Extracts were prepared by glass bead lysis in YEB (25 mM HEPES, pH 7.4, 150 mM NaCl, 0.2% NP-40, 5% glycerol, 0.5 mM DTT, 2 mM EDTA, 5 mM NaF, and protease inhibitors (as described)).

Preparation of Cdc6 Antibodies

Cdc6 antibody was generated to the C-terminal 330 amino acids of *CDC6* fused to *TrpE* and expressed in bacteria. Antibodies to TrpE were removed by passing serum over a TrpE affinity column.

Immunoprecipitations

12CA5 antibody was incubated for 1 h with extract prepared in YEB at a ratio of 1-5 μg of antibody per mg extract. 100 ml of 100 mg/ml protein A Sepharose slurry was added and incubated for an additional hour. Beads were washed extensively with YEB before using in kinase assays.

Protein Kinase Assays

Reactions for immunoprecipitated kinases and kinases copurifiying with GST-Cdc6 were carried out in 50 mM Tris-HCl, pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, 500 cpm/pmol [γ^{32}P]ATP for 30 min at 37°C. Samples were electrophoresed on a 10% denaturing gel and autoradiographed. Protein kinase A (PKA) was ordered from New England Biolabs, and assays were carried out as per manufacturer's instructions.

Complementation of a MET3-CDC6 Strain by CDC6 and cdc6DN

Plasmids expressing *GAL-CDC6, GAL-cdc6ΔN, CDC6* or *cd6cΔN* were transformed into K4055 (see Methods for description of plasmids and strain) and transformants were selected on medium lacking methionine and uracil (YEp352 series, *GAL1,10* promoter) or lacking methionine and leucine (pRS315 series, *CDC6* promoter). Colonies were purified on the same medium, then tested for their ability to grow on solid medium containing 2mM methionine. The *GAL1,10* promoter series were tested on medium containing glucose only, raffinose only, glucose and galactose, and raffinose and galactose as carbon sources. The *CDC6* promoter series was plated on medium containing glucose. All sugars were present at a concentration of 2%. Cells were grown on plates for three days, at which time both colonies and cells were photographed.

RESULTS

Cdc6 and Cdc28 Protein Interact

In order to study the molecular mechanisms by which Cdc6 participates in DNA replication, Cdc6 was purified from yeast as a GST-Cdc6 fusion protein (Figure 1A). The purified protein was assessed for biochemical activities that might be associated with a protein with the properties defined by genetic and molecular biological analysis of the *CDC6* gene. In particular, ATPase activity was investigated, but no ATPase activity could be demonstrated as intrinsic to the protein. Others have reported that an ATPase associates with bGST-Cdc6 purified from *E. coli* (Zwerschke *et al.*, 1994), and this discrepancy will be discussed elsewhere (Jong and Campbell, unpublished data). Given the pivotal role that Cdc6 is likely to play in the initiation of DNA replication, we next examined whether Cdc6 might interact with the Cdc28 protein kinase, whose action is necessary for the firing of replication origins (Epstein and Cross, 1992; Kuhne and Linder, 1993; Richardson *et al.*, 1992; Schwob and Nasmyth, 1993). As shown in the protein blot in Figure 1B, Cdc28 is indeed associated with the purified, recombinant Cdc6 protein. When glutathione agarose charged with GST-Cdc6 is washed with buffer containing 1.5M NaCl, bound Cdc28 can still be detected to the same degree (unpublished observations). Thus, the proteins appear to interact with very high affinity.

Since Cdc6 is overproduced in the experiments shown in Figure 1B, it was possible that the observed association was an artifact of overproduction. In order to show that Cdc6 interacts with Cdc28 at physiological concentrations, and to confirm that endogenous Cdc6, as well as the yGST-Cdc6 fusion protein, binds to Cdc28, the association was investigated in wildtype yeast cells, that is cells carrying neither tagged nor overproduced Cdc6 or Cdc28. Cdc28 was enriched on p13 agarose beads and the Cdc28/p13 beads were assessed for bound Cdc6 using polyclonal Cdc6 antibody. As shown in Figure 2, Cdc6 is associated with Cdc28.

To demonstrate that copurification shown in Figure 1 was due to interaction between the two proteins, hemagglutinin epitope-tagged Cdc28 (HA-Cdc28) and yGST-Cdc6 were coexpressed in yeast. yGST-Cdc6 was found in immunoprecipitates of HA-Cdc28 prepared with the anti-epitope antibody 12CA5 (Figures 3A), but not in 12CA5 immunoprecipitates in which tagged Cdc28 was absent (Figure 3B). Similarly, HA-Cdc28 was present in glutathione agarose purified yGST-Cdc6 but not in yGST preparations (Figure 3C). In a separate set of experiments, we had observed that mutation of lysine 114 to glutamate generated a Cdc6 protein that failed to complement the a *cdc6Δ* mutant (Jong and Campbell, unpublished). We asked whether this defect in growth was correlated with a lack of Cdc28 binding, and observed that the binding of Cdc28 to the mutant protein was equivalent to wild type (Figure 3A). Therefore, using diverse methodologies, we observed that the Cdc6 and Cdc28 proteins can form a stable and specific complex, though we estimate that only a small fraction of the total cellular Cdc28 is present in complexes with Cdc6.

The N-terminal 47 Amino Acids of Cdc6 Are Necessary and Sufficient for Cdc28 Binding.

To identify the region of the Cdc6 protein that is important for interaction with Cdc28, a deletion analysis was performed. Full length and truncated bGST-Cdc6 proteins were expressed in *E. coli* (Figure 4A), purified with glutathione agarose beads and incubated with yeast extracts in the presence or absence of ATP. After extensive washing, the bound proteins were eluted and analyzed by Western blotting. Cdc28 present in the yeast extract was clearly retained on the bGST-Cdc6 beads (Figure 4B, lane 1). This analysis revealed that the N-terminal 47 amino acids of Cdc6 are sufficient for interaction with Cdc28 (Figure 4B, lane 7). This region contains several Cdc28 recognition sites, two of which overlap a putative bipartite nuclear localization signal and one of which is adjacent

to a potential PEST sequence (Zhou *et al.*, 1989). The 330 amino acid C-terminal domain did not form a complex with Cdc28 (Figure 4B, lane 3). Interestingly, Cdc28 associated with the bGST-Cdc6 only in the presence of ATP. This dependence does not appear to reflect a requirement for the Cdc6 nucleotide binding site (Zhou *et al.*, 1989), since fusions lacking the nucleotide binding site (NP) still bind Cdc28. Furthermore, proteins with a mutation (K114E) in the consensus nucleotide binding domain (Jong and Campbell, unpublished) also interact with Cdc28 to the same extent as wildtype. Thus, the role of the ATP binding site does not appear to be to promote the interaction with Cdc28.

Binding of Cdc6 to Different Cyclin/Cdc28 Complexes

In *S. cerevisiae*, the major cell cycle transitions are accompanied by accumulation of different classes of cyclins: *CLN1-3*, non-B-type cyclins present in G1 and involved in commitment to the cell cycle and budding; *CLB5* and *6*, B-type cyclins active at G1/S through G2 and involved in the initiation of DNA replication and perhaps in prevention of re-replication in S, G2 and M; and *CLB1,2*, B-type cyclins active at G2/Mand required for passage through mitosis. Additional B-type cyclins, *CLB3,4*, are expressed during S, G2, and M. Since Cdc6 is required for specific cell cycle transitions and not continuously during the cell cycle, one might then expect a specific cyclin class to be present in the Cdc28/Cdc6 complexes. In order to test this, yGST-Cdc6 was immobilized on glutathione-beads and mixed with extracts expressing either HA-Cln2 or HA-Clb5. HA-Clb5 was retained on the beads while HA-Cln2 was not (Figure 5). HA-Clb2, like HA-Clb5, was also found to interact with yGST-Cdc6 in cells coexpressing the two tagged proteins (unpublished observations). Therefore, there is specific formation of B-type cyclin/Cdc28/Cdc6 complexes, suggesting that there is cell cycle specific binding of Cdc6 to Cdc28 and not simply non-specific binding.

Cdc6 is an In Vitro Substrate of Cdc28 Kinases

Although the stable association does not necessarily indicate that Cdc6 is a substrate of the kinase, the fact that there are three potential Cdc28 phosphorylation sites within the 47 N-terminal amino acids of Cdc6 suggested that Cdc6 may be phosphorylated by Cdc28 (Zhou *et al.*, 1989). As shown in Figure 3A, when affinity purified yGST-Cdc6 containing Cdc28 was incubated with [γ-^{32}P]ATP, yGST-Cdc6 but not yGST was efficiently phosphorylated. To confirm that the kinase activity was due to Cdc28, yGST-Cdc6 and yGST were expressed in the temperature sensitive mutant *cdc28-4*. As shown in Figure 6A, phosphorylation of yGST-Cdc6 purified from the strain was highly thermolabile. The levels of cdc28-4 protein in the Cdc6 preparation were equal to or greater than the levels of Cdc28 present when Cdc6 was purified from wild type cells. To confirm that the phosphorylated protein in Figure 6A was indeed Cdc6, bGST-Cdc6 was tested as a substrate of hemagglutinin epitope (HA) tagged Cdc28. HA-Cdc28 was immunoprecipitated from asynchronously growing yeast and incubated with bGST or bGST-Cdc6 and [γ-^{32}P]ATP. bGST-Cdc6 was phosphorylated, but not bGST (Figure 6B). As a preliminary assessment of the possible physiological significance of Cdc6 phosphorylation, cells were labeled with $^{32}P_i$, and Cdc6 was shown to be a phosphoprotein in vivo (Figure 6C). Phosphoamino acid determination showed the presence of phosphoserine (unpublished observations).

Since yGST-Cdc6 binds preferentially to B-type cyclin/Cdc28 complexes, it was of interest to test the specificity of the Cdc28 kinase phosphorylating Cdc6. 12CA5 immunoprecipitates were prepared from strains carrying HA-tagged Cln2, Clb5 or Clb2 protein and tested for ability to phosphorylate bGST-Cdc6. Since three putative Cdc28 phosphorylation sites and the Cdc28 interaction domain are located within the first 47 amino acids, bGST-NP, described in Figure 3, was tested first as substrate with normalized levels of Cln and Clb kinases. It is clear that bGST-NP is preferentially

phosphorylated by B-type cyclin/Cdc28 kinases (Figure 6D). The phosphorylated bands, labeled bGST-NP, were shown to cross-react with GST antibody. Since the Cdc6 antibody was prepared using a fusion protein missing the N-terminal 190 amino acids, the presence of Cdc6 amino acids could not be directly tested. Two additional potential Cdc28 phosphorylation sites are located near the N-terminus of the protein, and therefore bGST-BE (see Figure 3A) was also tested as a substrate. bGST-BE is not detectably phosphorylated by any of the kinases in our assays (Figure 6D), suggesting that the major cyclinB/Cdc28 phosphorylation sites are at the N-terminus of Cdc6.

Cdc6 Protein Inhibits HA-Clb2/Cdc28 Kinase

Constitutive expression of *CDC6* has been shown to delay cells in G2 and to cause bud hyperpolarization (Bueno and Russell, 1992), phenomena that also occur when the mitotic cyclins are downregulated in G2 (Lew and Reed, 1993). Cdc6 may therefore directly or indirectly inhibit the mitotic Cdc28 kinases. We observed that in vitro Cdc6 directly inhibited phosphorylation of histone H1 by Cdc28 kinase prepared using either p13-agarose (unpublished observations) or HA-Clb2 immunoprecipitates (Figure 6E). Since Cdc6 is also a substrate of B-type cyclin/Cdc28, the inhibition seen here may be competitive, and therefore only of physiological significance when the Cdc6 protein is present in excess (see Discussion). However, as shown below (Figure 8), the levels of Cdc6 used to inhibit the HA-Clb2 immunoprecipitates (Figure 6E) are only slightly higher than the levels of $p40^{Sic1}$ required to inhibit HA-Clb5 immunoprecipitates under the conditions of our assays.

$p40^{Sic1}$ Inhibits the Association of Clb5,6/Cdc28 and Cdc6 In Vivo and In Vitro

Since both Cdc6 and Clb5,6 are required for the initiation of replication (Epstein and Cross, 1992; Kuhne and Linder, 1993; Richardson *et al.*, 1992; Schwob and Nasmyth, 1993), we have investigated their association in greater detail. B-type cyclin dependent kinases are inhibited by $p40^{Sic1}$, which is expressed in late mitosis and early G1 (Schwob *et al.*, 1994). In late G1, $p40^{Sic1}$ is degraded by the *CDC34* -dependent pathway (Schwob *et al.*, 1994), leading to activation of Clb5/Cdc28 kinase. One possible role for the interaction of Cdc6 with Clb5/Cdc28 may be to trigger the conversion of the inactive origin into an active replication fork. In this case, $p40^{Sic1}$ may serve to prevent the interaction until the cell is completely prepared for S phase. To test the effect of intracellular $p40^{Sic1}$ levels on Cdc6/Cdc28 complex formation, a *cdc34* strain carrying the HA-tagged *CLB5* gene was blocked at the restrictive temperature, and the presence of Clb5 and its ability to associate with Cdc6 were assessed. While HA-Clb5 present in asynchronous cultures was able to bind to Cdc6, HA-Clb5 present in *cdc34* arrested cells, which cannot degrade $p40^{Sic1}$ and therefore contain high levels of $p40^{Sic1}$, did not bind (Figure 7). In contrast, in cells arrested in G2 with nocodazole, where $p40^{Sic1}$ is absent, HA-Clb5/Cdc28 did associate with Cdc6 (Figure 7). This suggested that $p40^{Sic1}$ not only inhibits the kinase activity of Clb5/Cdc28 but also inhibits the association of Clb5/Cdc28 with Cdc6.

To establish that the lack of binding in the *cdc34*-arrested cells was due to $p40^{Sic1}$, an MBP- $p40^{Sic1}$ fusion protein was purified (see legend to Figure 8). As shown in Figure 8B, the fusion protein was active in inhibiting Clb5/Cdc28 histone H1 kinase activity and also inhibited the association of Clb5/Cdc28 with the yGST-Cdc6 beads (Figure 8A). As a control for the specificity of the inhibition, protein kinase A was shown to be resistant to inhibition by the MBP- $p40^{Sic1}$ fusion protein (Figure 8C). Therefore, Clb5/Cdc28 does not bind to Cdc6 in the presence of $p40^{Sic1}$, and binding in vivo therefore probably occurs after degradation of $p40^{Sic1}$.

Effect of Deletion of the N-terminal 47 Amino Acids from CDC6

We showed above that the 47 N-terminal amino acids of *CDC6* are sufficient for interaction between Cdc6 and Cdc28. In order to evaluate the specific role of the Cdc28/Cdc6 interaction in vivo, the first 47 amino acids of *CDC6* were deleted. The phenotype of cells expressing only this *cdc6ΔN* mutant was evaluated using a *MET3-CDC6::TRP1cdc6Δ* strain. In the presence of methionine, this strain will only grow if a functional copy of *CDC6* is provided on a plasmid. Since the levels of Cdc6 protein are regulated dramatically in the cell cycle (Piatti *et al.*, 1995) and since the N-terminal 47 amino acids contain a PEST sequence that could be important for such regulation, we examined the ability of *cdc6ΔN* to complement the methionine repressed host not only under the control of the native promoter but also under several different expression regimes. When expressed from the *GAL* promoter on medium containing glucose plus galactose, the wildtype *GAL-CDC6* gene supported growth of the *MET-CDC6* strain in the presence of methionine (Figure 9). In contrast, the *GAL-cdc6ΔN* mutant did not support growth under these growth conditions. *GAL-cdc6ΔN* also failed to support growth on the non-repressing carbon source raffinose (unpublished observations), whereas the wildtype supported growth at normal rates. Assuming that both mutant and wildtype proteins are transcribed and translated at the same level, a reasonable assumption given our experience with the GST tagged constructs, these experiments suggest that the *cdc6ΔN* mutant is defective in some essential function of Cdc6.

A different result was obtained after induction of *GAL-CDC6* and *GAL-cdc6ΔN* with galactose. The overproduced cdc6ΔN protein is capable of supporting growth, suggesting that cdc6ΔN is less efficient than Cdc6 at promoting DNA replication, but can do so when present in excess. What is interesting in addition is that overproduction of wildtype *CDC6* leads to slow growth and bud hyperpolarization, as originally reported by

others (Bueno and Russell, 1992), but overproduction of *cdc6ΔN* results in growth at normal rates and the cells show normal morphology (Figure 10). It has been proposed that overproduction of wildtype Cdc6 leads to restraint of mitosis, and the results shown in Figure 10 suggest that the Cdc6/Cdc28 interaction is important for this inhibition of mitosis.

When *CDC6* and *cdc6ΔN* were expressed under control of the *CDC6* promoter, *cdc6ΔN* supported growth at the same rate as wildtype. Sufficient *cdc6ΔN* must be produced under these expression conditions so that initiation can occur. It may be the case that *cdc6ΔN* is present at elevated levels due to lack of the complete putative PEST sequence.

DISCUSSION

The yeast replication cycle model proposed recently based on genetic and physiological experiments posits that Clb5/Cdc28 is required both for activation of replication origins in G1 and for inhibition of re-activation of origins in S, G2 and M (Dahmann *et al.*, 1995; Piatti *et al.*, 1996). We have now shown that Cdc6 has all the right properties to be a critical target of Clb5/Cdc28 regulation. Cdc6 associates stably with Cdc28 in vivo. Cdc6 binds preferentially to Clb rather than Cln/Cdc28 complexes (Figure 5), and the two proteins are not associated before the *CDC34* -dependent step is completed in G1, presumably due to inhibition by $p40^{Sic1}$ (Figure 8). That the association of the Cdc6 and Cdc28 kinase is physiologically important is demonstrated by the effect of deleting the Cdc28 interacting domain from the Cdc6 protein. At low levels of expression, where wildtype Cdc6 supports cellular growth, the mutant fails to form colonies. At high levels of expression, the mutant can support growth. Thus, the mutant protein is less efficient than wildtype Cdc6 in promoting cell growth. In addition, the inhibition of growth and bud hyperpolarization seen when wildtype is overexpressed is not observed, suggesting that the Cdc28 interaction domain has an important role restraining mitosis and thus in ordering DNA replication and mitosis.

A model incorporating our results and those of others is shown in Figure 11. In vivo footprinting of ARSs has suggested that yeast replication origins alternate between at least two different states (Diffley *et al.*, 1994). During late mitosis and early G1, origins are in a pre-replicative state primed for initiation. Upon origin activation at G1/S, ARSs are converted to a post-replicative state, which is refractory to reinitiation and persists through S, G2 and part of M. The post-replicative complex (postRC) consists of ORC and ABF1 (Diffley *et al.*, 1994). Resetting to the pre-replicative complex (preRC) occurs late in mitosis coincident with the time of cyclin B degradation, $p40^{Sic1}$ expression, and Cdc6 expression (Diffley *et al.*, 1994; Donovan *et al.*, 1994; Piatti *et al.*, 1995). It is likely that a

key step in the transition involves recruitment of Cdc6 into the ORC/ABF1 complex, since formation of preRCs requires Cdc6, since preRCs are thermolabile in *cdc6^{ts}* mutants, and since Cdc6 shows both genetic interactions with ORC and interacts stably with ORC in vitro (Cocker *et al.*, 1996, and see Figure 5; Diffley *et al.*, 1994; Li and Herskowitz, 1993; Liang *et al.*, 1995). Cdc6 may in turn recruit other replication proteins, such as the Mcm family (Yan *et al.*, 1993). Three key observations suggest that the putative recruitment of Cdc6 into the preRC is regulated by cyclin B/Cdc28. First, formation of the preRC can be brought about by premature expression in G2 of the Clb5/Cdc28 inhibitor, p40^{Sic1}. Second, in *sim1* and *sim2* mutants, which undergo a second round of replication when arrested in G2, cyclin B/Cdc28 kinase levels are very low and overproduction of Clb5, rather than stimulating initiation, actually prevents re-replication (Dahmann *et al.*, 1995). Third, there exists a "point of no return" in G1, after which Cdc6 cannot be recruited into the preRC and this "point of no return" coincides temporally with the degradation of p40^{Sic1} and activation of Clb5/Cdc28 (Piatti *et al.*, 1996). Our demonstration that Cdc6 interacts biochemically with Clb5/Cdc28 and that the interaction is inhibited by p40^{Sic1}, which has now also in part been shown by others (Piatti *et al.*, 1996) suggests that Cdc6 is a target of Clb5/Cdc28 and that Clb5/Cdc28 inhibits Cdc6 recruitment into the preRC. The *cdc6ΔN* mutant we describe (Figure 9) may be able to function when present in excess but not at lower levels, because there is a longer window for formation of the preRC, continuing even after activation of Clb5/Cdc28 and providing time for a less efficient activator to function. Only after cyclin B degradation and p40^{Sic1} expression late in mitosis can the preRC form. Our results taken together argue for a post-translational role for the kinase/Cdc6 interaction, rather than indirect effects on Cdc6 mRNA expression. A prediction of this model is that Cdc6 binding to Clb5/Cdc28 should prevent binding of Cdc6 to the ORC/ARS complex.

The proteins in the preRC are poised for origin firing, but initiation requires further activation in G1 by S phase promoting factors. Both Cdc7/Dbf4 kinase (Rowley *et al.*, 1995) and Clb5,6/Cdc28 kinase (Schwob and Nasmyth, 1993) are required and probably phosphorylate proteins in the preRC, such as ORC (Leatherwood *et al.*, 1996). The interaction of Clb5/Cdc28 with Cdc6 in late G1 may also be a key step in origin activation, since deletion of the Cdc28 interaction domain leads to defects in growth. A sudden conversion of the preRC into the postRC (which appears to contain ORC but not Cdc6) is observed at the beginning of S phase and is thought to coincide with origin activation. Interaction of Cdc6 with Clb5/Cdc28, which occurs only after $p40^{Sic1}$ degradation, might lead to the transition. Phosphorylation may or may not be required. Clb5/Cdc28 might extract Cdc6 from the complex with ORC and sequester the Cdc6 or target it for degradation. The latter model is attractive, since Cdc6 protein has a short half-life. However, degradation of Cdc6 has not yet been shown to be essential for entry into S phase (Piatti *et al.*, 1996). In this way, activation of Clb5/Cdc28 might lead not only to firing of primed origins, perhaps by relieving some inhibitory function of Cdc6, but also to suppression of de novo preRC formation both immediately following origin duplication and until cyclin B destruction at mitosis. Interaction of Cdc6 with Clb5/Cdc28 could therefore account for once and only once replication and preservation of the order of replication and segregation of chromosomes, i.e., prevention of re-replication without an intervening mitosis.

When constitutively expressed at high levels, Cdc6 can transiently inhibit mitosis (Bueno and Russell, 1992) and slow the growth of yeast (this work). Our demonstration that high levels of Cdc6 inhibit Clb2/Cdc28 (Figure 6) in vitro could account for the G2 delay observed when Cdc6 is overproduced and therefore present in excess or for a long time. Levels of Cdc6 we observe in vivo after four hours of expression are comparable to concentrations used in our assays. During normal cell cycles, Cdc6 is not likely to regulate

the G2/M transition by such direct inhibition, primarily because Cdc6 levels are either quite low or absent at this phase of the cell cycle. Other observations, however, suggest that Cdc6 at normal concentrations may play a more indirect role in restraining mitosis. Rao and Johnson showed that when a G2 cell is fused to an S phase cell, the G2 nucleus does not enter mitosis until the S phase nucleus has also completed DNA synthesis and traversed G2 (Rao and Johnson, 1970). It has been proposed that cells in S phase generate a signal that restrains mitosis. The Cdc6/cyclin B/Cdc28 complex might constitute such a signal. The difference between the phenotype of cells overexpressing wildtype *CDC6* and the *cdc6ΔN* truncation mutant that does not interact with Cdc28 support this idea (Figure 10). Therefore, destruction of Cdc6 may be required for entry into mitosis.

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Figure 1. Cdc28 copurifies with Cdc6.

(A) Electrophoretic analysis of yGST and yGST-Cdc6 expressed in yeast and affinity purified on glutathione agarose. Glutathione-S-transferase (GST) and GST-tagged Cdc6 were expressed in yeast and purified as described (see Methods). Purified yGST-Cdc6 (100 ng) was eletrophoresed, stained with silver (lane 1), and analyzed by immunoblotting with Cdc6 antibody (lane 2) and GST antibody (lane 3). Cdc6 antibody is described in Methods.

(B) Cdc28 copurifies with yGST-Cdc6. Purified yGST and yGST-Cdc6 protein were electrophoresed, blotted and probed with Cdc28 antibody (gift of R. Deshaies, California Institute of Technology). Lane 1, 100 μg extract; lane 2, 100 ng yGST; lane 3, 100 ng yGST-Cdc6.

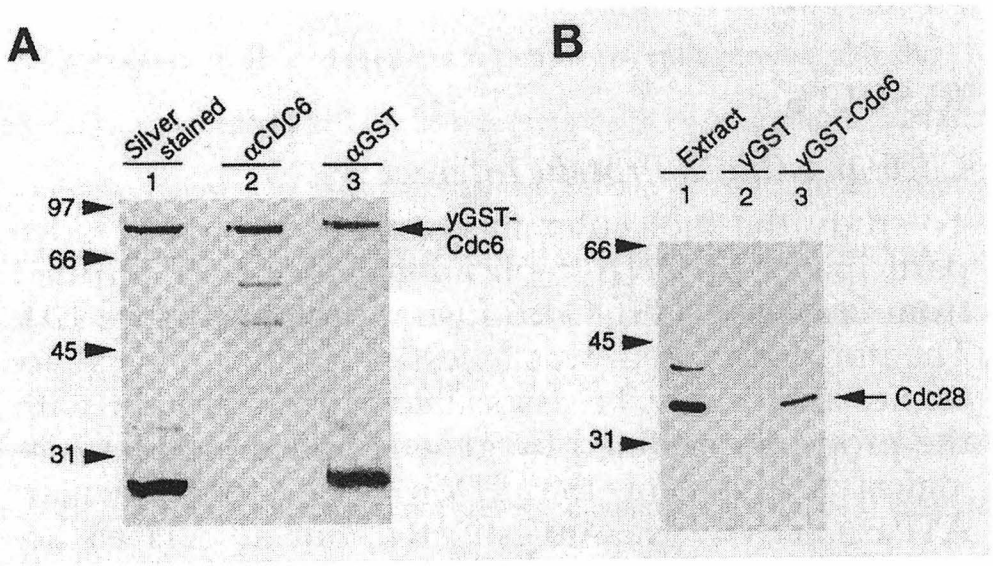

A
Silver stained
αCDC6
αGST
1
2
3
97
66
45
31
yGST-Cdc6
B
Extract
yGST
yGST-Cdc6
1
2
3
66
45
31
Cdc28

Figure 2. Association of endogenous Cdc6 with endogenous Cdc28. 10 mg of BJ2168 extract was prepared and Cdc28 was precipitated by incubating extract with 20 ml of p13 agarose, followed by extensive washing. Protein was eluted, electrophoresed and immunoblotted with Cdc6 antibody. Lane 1, 1 μg of conventionally purified, nontagged Cdc6 (to be described elsewhere); lanes 2-4, p13-affinity purified Cdc28. Immunoblots were prepared as follows: lanes 1-2 were probed with Cdc6 antibody; lane 3 was probed with Cdc6 antibody but secondary antibody was omitted; lane 4 was probed with nonspecific antiserum. Cdc6 was not present on control beads (no p13) incubated with extract (unpublished observations).

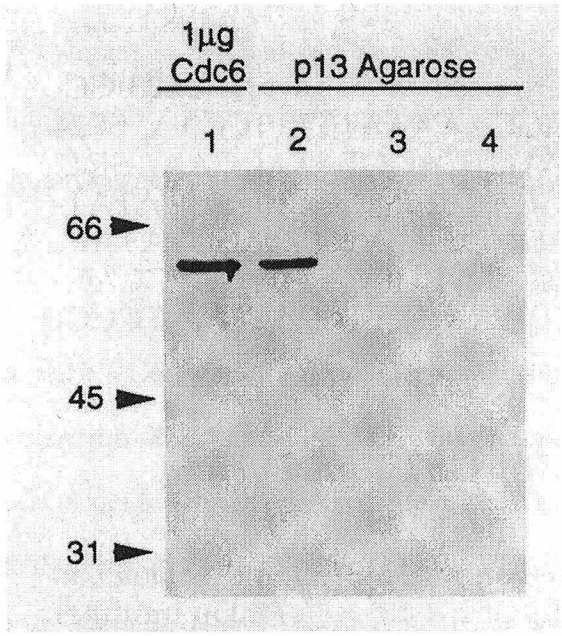

1µg
Cdc6
p13 Agarose
1
2
3
4
66
45
31

Figure 3. Association of Cdc28 with Cdc6 evaluated by coexpressing yGST-Cdc6 and HA-Cdc28 in yeast. yGST, yGST-Cdc6/K114E and yGST-CDC6 were expressed in strain RJD635, containing hemagglutinin-tagged Cdc28. GST-Cdc6/K114E was generated by mutating lysine 114, which falls in the putative nucleotide binding motif, GTGKT, to glutamate. This mutant does not complement a deletion of cdc6 (Jong and Campbell, unpublished). Extracts were prepared, and 1 mg samples were used for 12CA5 immunoprecipitations and glutathione agarose purifications, omitting the elution step.

(A) Cdc28 copurifies with yGST-Cdc6. Immunoprecipitates and glutathione agarose purified protein were run on 8% and 12% SDS gels and transferred to Immobilon. The blot of the 8% gel was probed with GST antibody (top panel), and the blot of the 12% gel was probed with Cdc28 antibody (bottom panel). Lanes 1 and 2, 8% gel, 30 μg extract; lanes 1 and 2, 12% gel, 3 μg extract; lanes 3 and 4, 12CA5 immunoprecipitates; lanes 5 and 6, glutathione agarose-bound protein. Odd numbered lanes; yGST-Cdc6/K114E; even numbered lanes, yGST-Cdc6.

(B) anti-GST immunoblot showing that GST-Cdc6 is not precipitated by 12CA5 when expressed in a strain lacking HA tagged Cdc28. Lane 1, 25μg extract with GST-Cdc6 expressed in BJ2168; lane 2, 12CA5 immunoprecipitation from 2mg of the same extract.

(C) 12CA5 immunoblot showing that Cdc28 is only present in glutathione agarose purified yGST-Cdc6 and not in purified GST. Lane 1, purified yGST; lane 2, purified yGST-Cdc6, as in Figure 3A, lane 6.

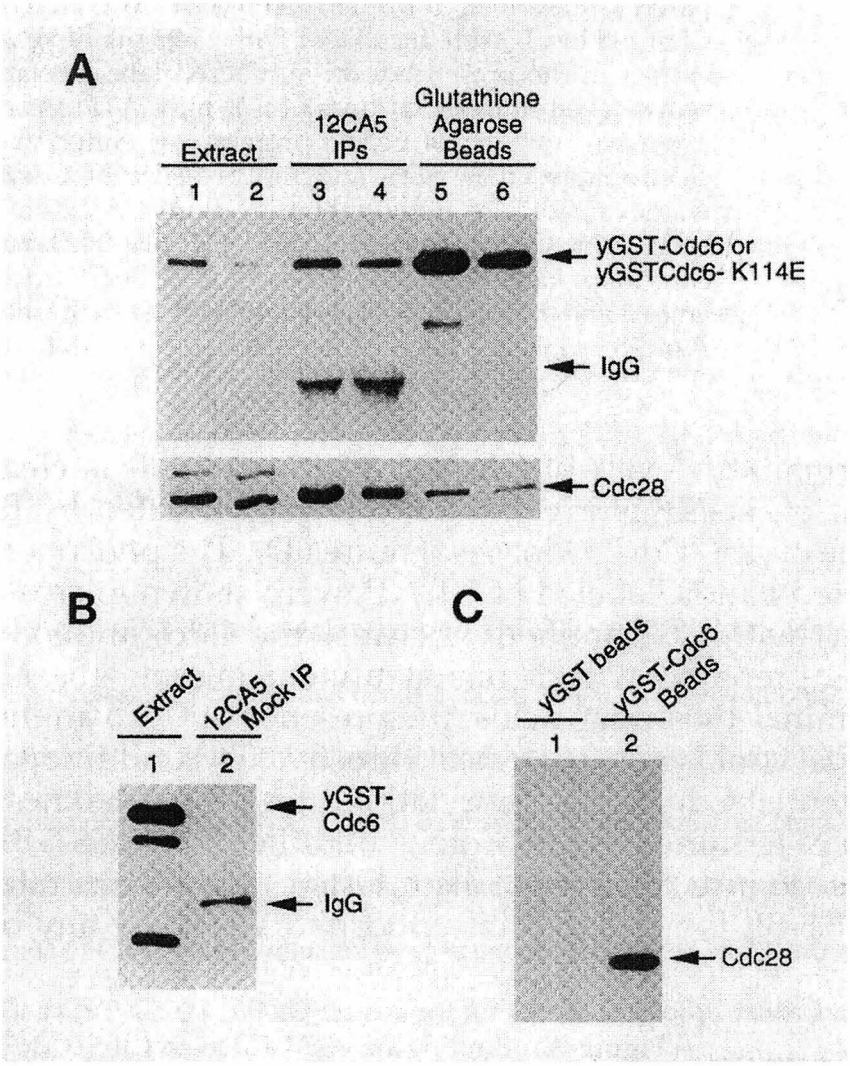

A
Extract
12CA5 IPs
Glutathione Agarose Beads
1 2 3 4 5 6
yGST-Cdc6 or yGSTCdc6- K114E
IgG
Cdc28
B
Extract
12CA5 Mock IP
1 2
yGST-Cdc6
IgG
C
yGST beads
yGST-Cdc6 Beads
1 2
Cdc28

Figure 4. Mapping of the region of Cdc6 required for binding Cdc28.

(A) Schematic of the *CDC6* truncations and summary of binding. Cross-hatched region, GST tag; vertical black lines, putative Cdc28 phosphorylation sites; black box, nucleotide binding site. Constructs: bGST-Cdc6, GST fused to full length *CDC6* ; bGST-NB, GST fused to residues 1-190 of *CDC6*; bGST-NP, GST fused to residues 1-47 of *CDC6*; bGST-BE, GST fused to residues 191-513 of *CDC6*.

(B) Binding of bGST-Cdc6 derivatives to Cdc28. bGST-Cdc6 and mutant proteins were expressed in bacteria and beads bearing 10-20 μg of fusion proteins were prepared, as described in Methods. Charged beads were incubated with 11.25 mg yeast extract in the presence (odd numbered lanes) or absence (even numbered lanes) of 1 mM ATP. Following extensive washing of beads, protein was eluted, electrophoresed in SDS, and the presence of Cdc28 was evaluated by immunoblotting with Cdc28 antibody. Lane C, 1 μg purified GST-Cdc28 (60 kD) as control for Cdc28 antibody; lanes 1 and 2, bGST-Cdc6; lanes 3 and 4, bGST-BE; lanes 5 and 6, bGST-NB; lanes 7 and 8, bGST-NP; lanes 9 and 10, bGST-Cdc6/K114E.

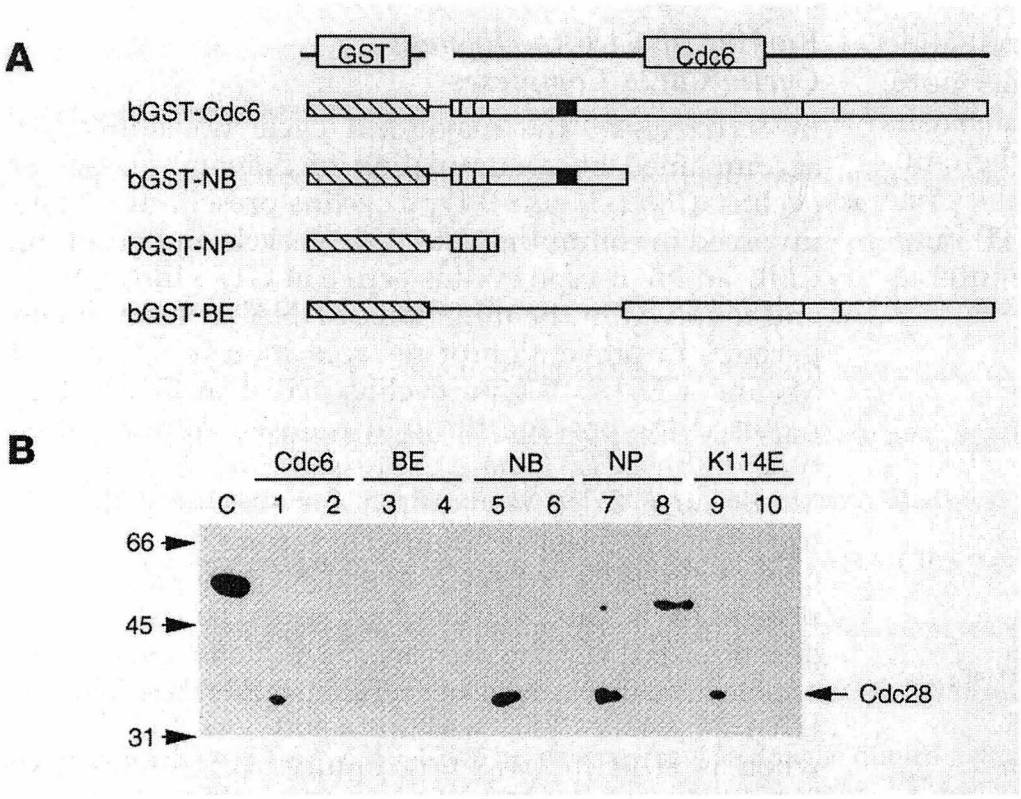

A
GST
Cdc6
bGST-Cdc6
bGST-NB
bGST-NP
bGST-BE
B
Cdc6
BE
NB
NP
K114E
C 1 2 3 4 5 6 7 8 9 10
66
45
31
Cdc28

Figure 5. Binding of yGST-Cdc6 to Clb5/Cdc28 and not to Cln2/Cdc28. yGST and yGST-Cdc6 were expressed in yeast as described in Methods and shown in Figure 1. Cdc6-containing beads were prepared by incubating extract with glutathione agarose for two hours followed by washing as described for purification of fusion proteins from yeast. Beads equivalent to 2 mg of extract were then incubated with extracts of cells expressing HA-tagged Clb5 or HA-tagged Cln2 and 2 mM ATP (Peter and Herskowitz, 1994). Strain RJD934 was used as a source of tagged Cln2. As shown by the 12CA5 Westerns on the left, the amount of extract of cells expressing equivalent amounts of HA-Cln2 or HA-Clb5 was determined, and the amount of extract incubated with beads was adjusted to contain approximately equal levels of HA-Cln2 (top panel) or HA-Clb5 (bottom panel). For HA-Cln2, four concentrations ranging from 0.08 to 10 mg protein/ml were used for binding, and for HA-Clb5, four concentrations ranging from 0.02 to 2.0 mg protein/ml were used. yGST- or yGST-Cdc6 beads were incubated in the diluted extracts and then washed, and the amount of cyclin retained was determined by immunoblotting. Lanes 1-4 (top and bottom panels), 10μl of extracts at increasing concentration. Lane E (top panel only) is an extract identical to lane 4, and was used as positive control for the HA-Cln2 Western after binding the Cdc6-beads. Lanes 5-8 (top and bottom panels), tagged cyclin retained by yGST beads incubated in increasingly concentrated extract; lanes 9-12 (top and bottom panels), tagged cyclin retained by yGST-Cdc6 beads incubated in increasingly concentrated extract.

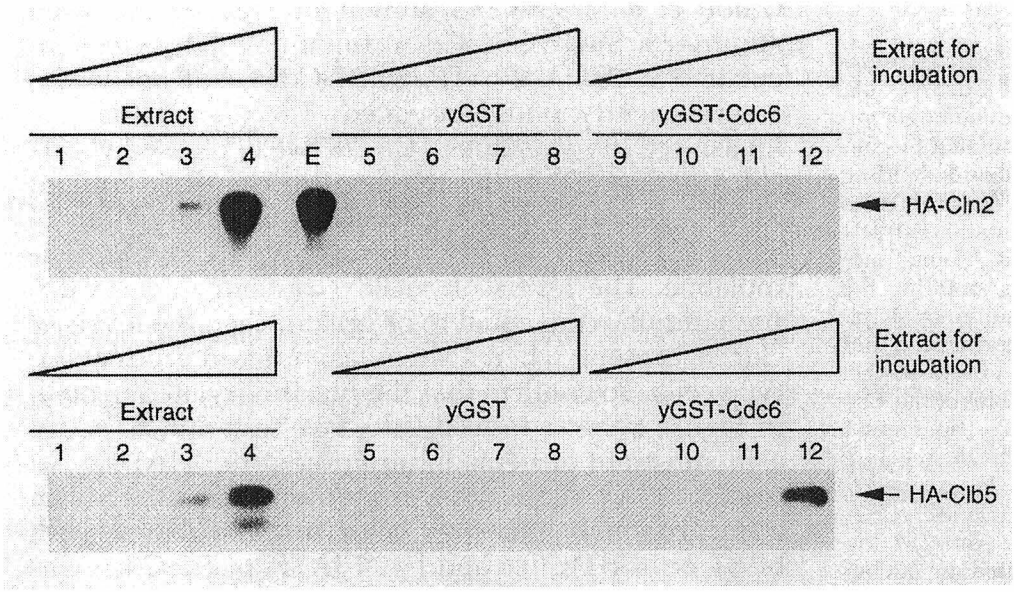

Extract for incubation
Extract
yGST
yGST-Cdc6
1 2 3 4 E 5 6 7 8 9 10 11 12
HA-Cln2
Extract for incubation
Extract
yGST
yGST-Cdc6
1 2 3 4 5 6 7 8 9 10 11 12
HA-Clb5

Figure 6. Cdc6 is a substrate for Cdc28 kinase and a phosphoprotein in vivo.

(A) Cdc28 purifies with and phosphorylates Cdc6. yGST and yGST-Cdc6 were expressed as before and purified from wild type (BJ2168) and *cdc28-4* (RJD561) strains. Purified proteins were assayed for associated kinase activity at 30°C, 37°C and 42°C (see Methods). The products of the kinase assay were run on a 10% polyacrylamide gel containing SDS and autoradiographed. Lanes 1-6, protein purified from wild type cells; lanes 7-12, protein purified from *cdc28-4* cells. Lanes 1-3 and 7-9, 200 ng yGST; lanes 4-6 and 10-12, 100 ng yGST-Cdc6. Lanes 1, 4, 7 and 10 assayed at 30°C; lanes 2, 5, 8 and 11 assayed at 37°C; lanes 3, 6, 9 and 12 assayed at 42°C.

(B) Bacterially produced Cdc6 is a substrate for Cdc28. bGST and bGST-Cdc6 were expressed in bacteria and purified. Kinase assays were performed with hemagglutinin epitope-tagged Cdc28 immunoprecipitated from RJD635 extracts with 12CA5 antibody. Mock immunoprecipitates were prepared with extract lacking epitope-tagged Cdc28. Lanes 1 and 2, mock immunoprecipitates prepared with 5 mg extract; lanes 3 and 4, 12CA5 immunoprecipitates prepared with 5 mg extract. Lanes 1 and 3, 500 ng bGST; lanes 2 and 4, 500 ng bGST-Cdc6. The band labeled p40 has not been verified as $p40^{Sic1}$.

(C) Cdc6 is phosphorylated in vivo. Log phase cells were labeled with inorganic $^{32}P_i$ (Yoon and Campbell, 1991). Cdc6 immunoprecipitates were prepared from cell extract. Proteins were eluted, electrophoresed and autoradiographed. Lane 1, mock immunoprecipitate with non-immune rabbit serum; lane 2, Cdc6 immunoprecipitate. Potato acid phosphatase decreased the label, showing it was due to phosphorylation (unpublished observation).

(D) The major region phosphorylated in Cdc6 is within the N-terminal 47 amino acids. The fusion proteins, bGST-NP and bGST-BE, were purified from bacteria as from yeast as described in Methods. The purity of the proteins was equivalent to that shown in Figure 1. Lanes 1, 3, and 5, bGST-NP as substrate for the indicated kinases. Lanes 2, 4, and 6, bGST-BE (see Figure 3) as substrate for the indicated kinases.

(E) Cdc6 inhibits HA-Clb2/Cdc28 histone H1 kinase activity. yGST-Cdc6 and yGST were prepared as above. HA-Clb2/Cdc28 was immunoprecipitated from 50 mg extract and assayed for histone H1 kinase in the presence of buffer (lane 1), 300 ng yGST (lane 2), or 300 ng yGST-Cdc6 (lane 3).

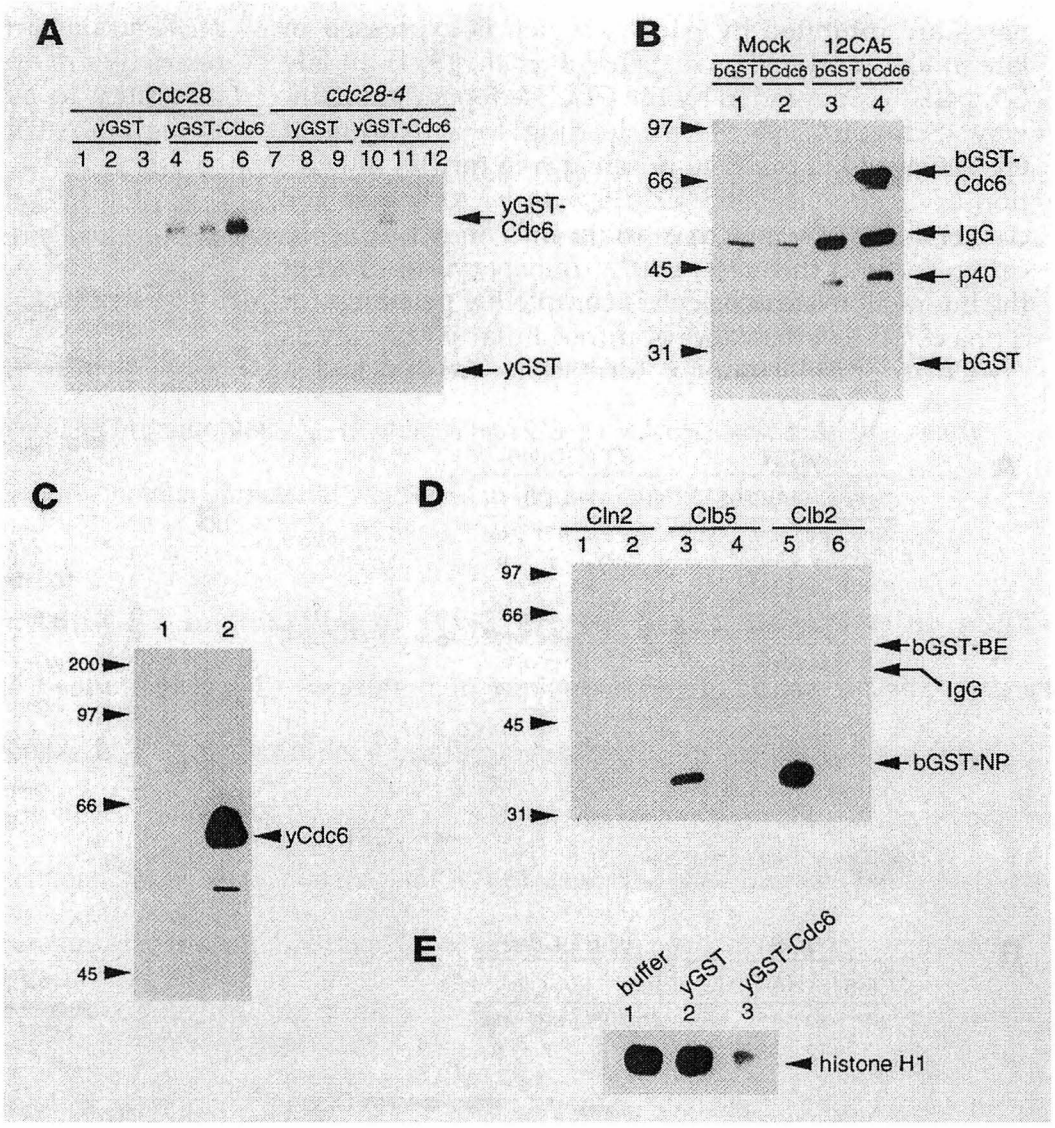

A
Cdc28
cdc28-4
yGST
yGST-Cdc6
yGST
yGST-Cdc6
1 2 3 4 5 6 7 8 9 10 11 12
yGST-Cdc6
yGST
B
Mock
12CA5
bGST bCdc6 bGST bCdc6
1 2 3 4
97
66
45
31
bGST-Cdc6
IgG
p40
bGST
C
1 2
200
97
66
45
yCdc6
D
Cln2
Clb5
Clb2
1 2 3 4 5 6
97
66
45
31
bGST-BE
IgG
bGST-NP
E
buffer
yGST
yGST-Cdc6
1 2 3
histone H1

Figure 7. Clb5/Cdc28 binds to Cdc6 only in the absence of p40^{Sic1}. The experimental conditions were as in Figure 5. Beads charged with yGST or yGST-Cdc6 were incubated with extracts expressing HA-Clb5 and blocked at either the *cdc34* step or the nocodazole step in the cell cycle. Beads were washed and analyzed by SDS gel analysis and immunoblotting. (Panels A and C are controls, indicating that nearly equivalent amounts of material were used in these experiment in panel B.)

(A) Anti-GST immunoblot of yGST protein beads (lanes 1-4) or yGST-Cdc6 beads (lanes 5-8) incubated in various extracts. Lanes 1 and 5, extracts of RJD668 (*cdc34-2*) incubated for 4 h at the nonpermissive temperature; lanes 2 and 6, extracts of JCY61 (*cdc34-2* , *ADH-CLB5-HA*) (see Methods) cycling at the permissive temperature; lanes 3 and 7, extracts of JCY61 incubated for 4 h at the nonpermissive temperature; lanes 4 and 8, strain JCY61 arrested with nocodazole at the permissive temperature.

(B) 12CA5 immunoblot of HA-Clb5 protein bound to bGST and bGST-Cdc6 beads (lanes the same as in panel A).

(C) Control 12CA5 immunoblot blot showing constitutive expression of HA-Clb5 in all JCY61 extracts.

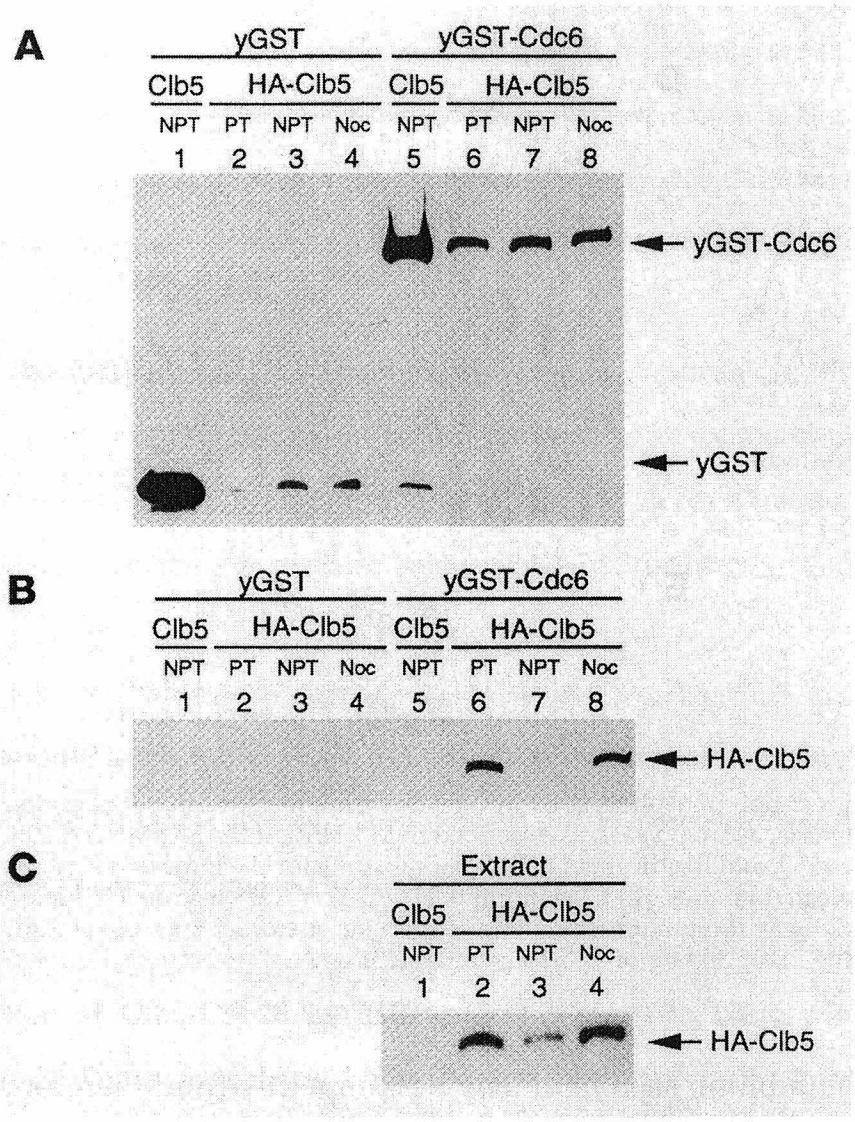

A
yGST
yGST-Cdc6
Clb5
HA-Clb5
Clb5
HA-Clb5
NPT
PT
NPT
Noc
NPT
PT
NPT
Noc
1
2
3
4
5
6
7
8
yGST-Cdc6
yGST
B
yGST
yGST-Cdc6
Clb5
HA-Clb5
Clb5
HA-Clb5
NPT
PT
NPT
Noc
NPT
PT
NPT
Noc
1
2
3
4
5
6
7
8
HA-Clb5
C
Extract
Clb5
HA-Clb5
NPT
PT
NPT
Noc
1
2
3
4
HA-Clb5

Figure 8. $p40^{Sic1}$ inhibits association of HA-Clb5 and Cdc6. Maltose binding protein (MBP) was fused to $p40^{Sic1}$. MBP and MBP- $p40^{Sic1}$ were expressed in *E. coli* and purified to homogeneity. The protein concentration was determined by Bradford analysis. The purified fusion protein was the gift of Dr. R. Deshaies, California Institute of Technology.

(A) 0.25 mg of HA-Clb5 extract was incubated with increasing amounts of MBP or MBP- $p40^{Sic1}$ (0, 80 ng, 400 ng, 2 μg). Preincubated extracts were then further incubated with bGST-Cdc6 beads in the presence of 2 mM ATP for 2 h. Beads were washed and analyzed for HA-Clb5 binding by protein blotting with 12CA5 antibody.

(B) Inhibition of Clb5/Cdc28 activity by MBP-$p40^{Sic1}$. HA-Clb5 extract was incubated with MBP and MBP-Sic1 as in (A). HA-Clb5 was immunoprecipitated and assayed for histone H1 kinase activity. Open symbols, MBP; closed symbols, MBP-p40; protein concentration was determined by Bradford assay.

(C) Inhibition of Clb5/Cdc28 by MBP-$p40^{Sic1}$ is specific. Histone H1 kinase activity of HA-Clb5 immunoprecipitated from 0.2mg of extract was assayed in the presence of increasing amounts of MBP (ranging from 0 to 2μg, first set of three lanes) and increasing amounts of MBP- $p40^{Sic1}$ (ranging from 0 to 2μg, second set of three lanes). Histone H1 kinase activity of protein kinase Awas assayed in the presence of increasing amounts of MBP (ranging from 0 to 2μg, third set of three lanes) and increasing amounts of MBP-$p40^{Sic1}$ (ranging from 0 to 2μg, fourth set of three lanes). Products were eletrophoresed and autoradiographed. Phosphorylated histone H1 is indicated.

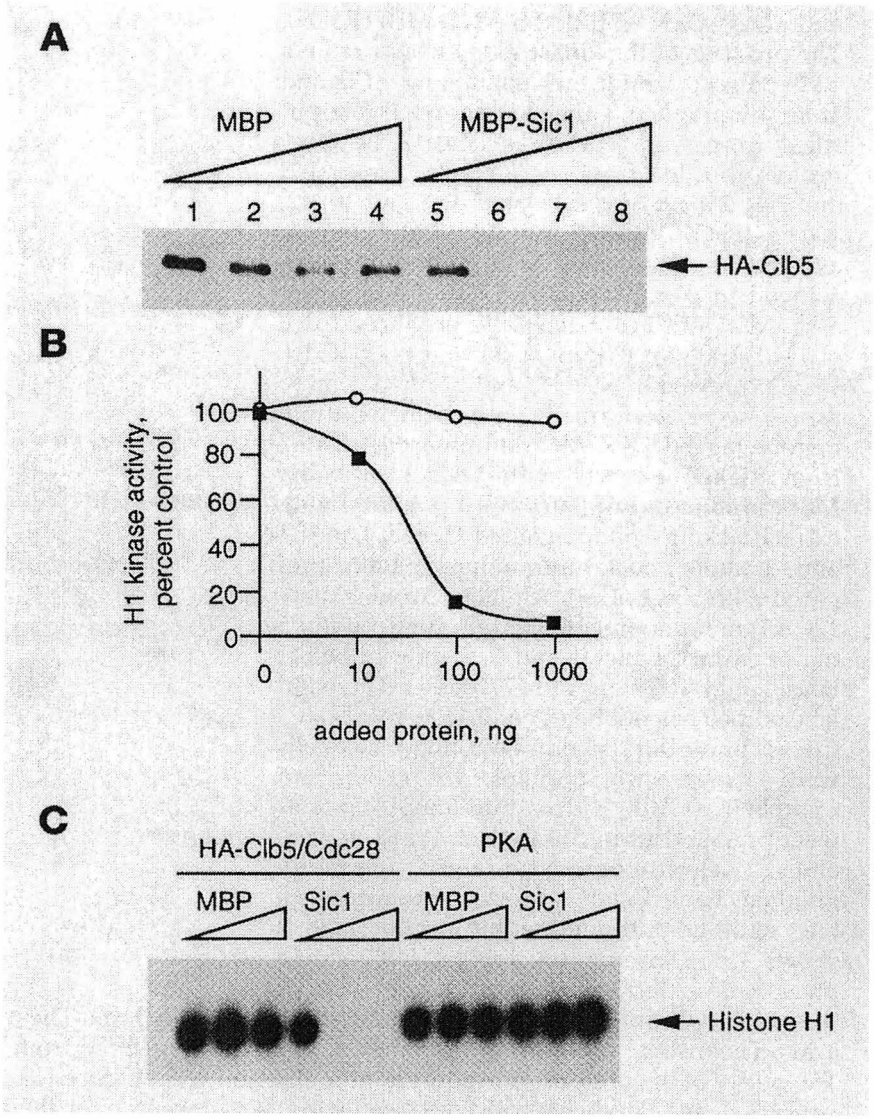

A
MBP
MBP-Sic1
1 2 3 4 5 6 7 8
HA-Clb5
B
H1 kinase activity, percent control
100
80
60
40
20
0
0 10 100 1000
added protein, ng
C
HA-Clb5/Cdc28
PKA
MBP
Sic1
MBP
Sic1
Histone H1

Figure 9. *Cdc6ΔN* complements a *cdc6* null only at elevated levels. Strain K4055 was transformed with plasmids YGp618 and pSE19, expressing *GAL-CDC6* and *GAL-cdc6ΔN* protein, respectively. The plasmid YGp10, carrying the *GAL1,10* promoter only, was used as a control. Transformants were streaked onto medium either lacking or containing 2mM methionine, and containing 2% dextrose and 2% galactose (*GAL* repressing conditions) or 2% raffinose and 2% galactose (*GAL* inducing conditions). Plates were incubated at 30°C for 3 days, then photographed.

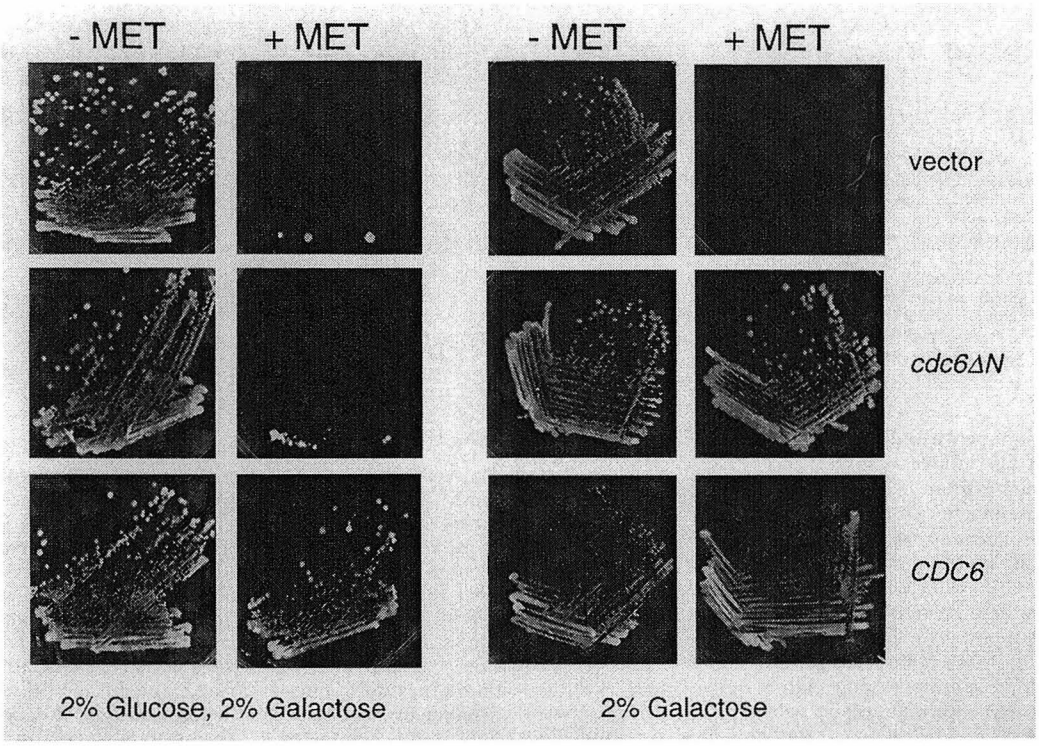

- MET
+ MET
- MET
+ MET
vector
cdc6ΔN
CDC6
2% Glucose, 2% Galactose
2% Galactose

Figure 10. Overproduction of full length but not cdc6ΔN protein perturbs cell growth and morphology. Colonies were picked from plates pictured in Figure 9 and cells photographed under a light microscope. Left panel, K4055 transformed with pSE19, *GAL-cdc6ΔN*, and grown on medium with 2mM methionine, 2% raffinose and 2% galactose. Right panel, K4055 transformed with YGp618, *GAL-CDC6*, and grown on the same medium.

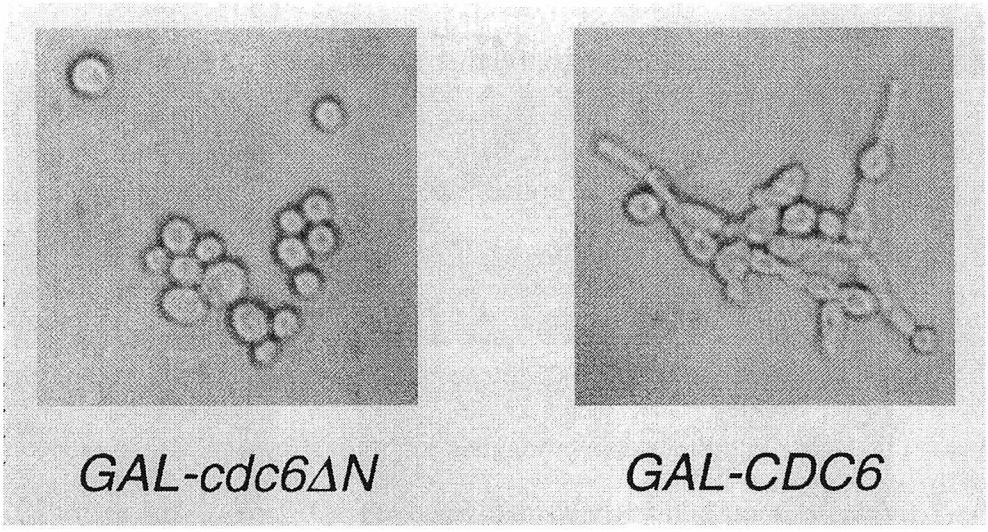
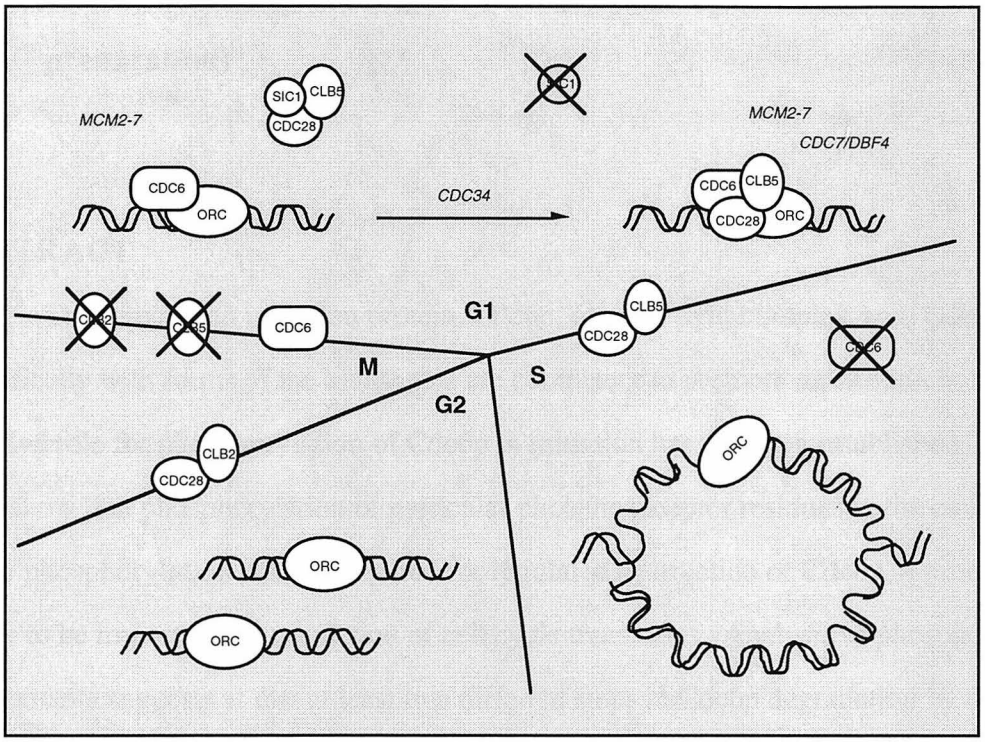

GAL-cdc6ΔN
GAL-CDC6

Figure 11. The Cdc6 cycle. After the mitotic cyclins are degraded in late mitosis, Cdc6 binds to ORC at replication origins in a step required to recruit additional replication proteins, such as Mcms, to the origins for initiation (formation of the preRC) (Diffley *et al.*, 1994). In G1, $p40^{Sic1}$ binds to Clb5/Cdc28 (Schwob *et al.*, 1994), preventing the kinase from binding to Cdc6. When $p40^{Sic1}$ is degraded by the *CDC34* dependent pathway in late G1, Clb5/Cdc28 associates with Cdc6 in the preRC, and perhaps with other components as well. This leads to origin activation, dissociation of Cdc6, and conversion of preRCs to postRCs. (PostRCs are found at this earliest portion of S phase.) At this time and until mitosis, Cdc6 is prevented from reassociating with the origin through interaction with B-type cyclin/Cdc28. This interaction may lead to degradation, sequestration or inactivation of an activity of Cdc6 (Nishitani and Nurse, 1995; Piatti *et al.*, 1995). Cdc6/cyclin B/Cdc28 might also form a signal for preventing mitosis. In late mitosis, cyclins are degraded and *CDC6* mRNA and protein accumulate; Cdc6 binds to ORC to form the preRC and primes the origin for the next round of replication.


MCM2-7
SIC1
CLB5
CDC28
SIC1
MCM2-7
CDC7/DBF4
CDC6
ORC
CDC34
CDC6
CLB5
CDC28
ORC
CLB2
CLB5
CDC6
G1
M
S
G2
CLB5
CDC28
CDC6
CLB2
CDC28
ORC
ORC
ORC

Chapter 3: Phosphorylation of Cdc6p Targets It for Rapid Destruction

(Suzanne Elsasser, Yong Chi, Ping Yang, and Judith L. Campbell, in preparation)

ABSTRACT

The replication initiation protein, Cdc6p, forms a tight complex with Cdk1p, specifically with forms of the kinase that are competent to promote replication initiation. A positive role for phosphorylation of Cdc6p in initiation has not been established, but we now show that phosphorylation of particular phosphoacceptor residues in the canonical Cdk1 phosphorylation sites is required for regulated destruction of Cdc6p, a process that is likely to be important for regulation of cell cycle transitions. Analysis of phosphorylation site mutants suggests at that at least two different steps in Cdc6p degradation by the Cdc34 ubiquitin-proteasome pathway are regulated by Cdk1p. First, phosphorylation of N-terminal sites targets Cdc6p for polyubiquitination probably through promoting interaction with Cdc4p, an F box protein involved in substrate recognition by the Cdc53/Skp1 ubiquitin ligase. Second, phosphorylation of a C-terminal site is required for a step apparently downstream of or parallel to ubiquitination. Ubiquitination appears to be mediated by Clb/Cdk complexes and not by Cln/Cdks, suggesting that phosphorylation controls the timing of degradation. The significance of degradation for cell-cycle control remains unclear. Stabilized mutants grow normally in wildtype backgrounds. However, overexpression of a non-phosphorylatable Cdc6p results in dominant lethality in a mutant affecting Cdc16p, a component of the APC/cyclosome.

INTRODUCTION

A hallmark of eukaryotic DNA replication is that replication is restricted to one phase of the cell cycle, S phase, and that this replication is mediated by the coordinated firing of a multitude of replication origins spread over many chromosomes. In *Saccharomyces cerevisae*, replication is initiated from hundreds of origins, and initiation depends on the Origin Recognition Complex (ORC), which can be detected at origins by means of in vivo footprinting and is present at origins throughout the cell cycle (Bell *et al.*, 1993; Bell and Stillman, 1992; Diffley and Cocker, 1992; Rowley *et al.*, 1995). At the end of mitosis the origin footprint expands, and genetic and molecular evidence suggest that the six-subunit ORC complex, Cdc6p, the six Mcms and Cdc45p are all assembled at the origins at this time (Newlon, 1997). Origins are then said to be licensed for replication (Blow and Laskey, 1988), and are subsequently activated by two kinases, Dbf4p/Cdc7p and Clb5,6p/Cdk1p. After this transition, the origin footprint returns to its prelicensed state.

In order to maintain genomic integrity, cells must coordinate DNA replication in such a way that every origin of replication fires no more than once per cell cycle. In *Saccharomyces cerevisiae*, the S phase cyclin/Cdks, Clb5p/Cdk1p and Clb6p/Cdk1p, promote the initiation of DNA replication and also prevent aberrant reinitiation. The S phase cyclin/Cdk proteins appear well before the G1/S transition, and are inhibited by the presence of Sic1p (Dahmann *et al.*, 1995; Dirick *et al.*, 1995; Nasmyth, 1996; Nasmyth, 1993; Schwob *et al.*, 1994; Schwob and Nasmyth, 1993; Surana *et al.*, 1993). Destruction of Sic1p at the G1/S transition activates the S phase cyclin/Cdks and DNA replication follows closely upon activation, occurring earlier in the cell cycle if Sic1p is deleted and the S phase cyclin/Cdks prematurely activated, and occurring later in the presence of a stabilized allele of Sic1p which maintains the S phase cyclin/Cdks in an inactive state (Schwob *et al.*, 1994). Additionally, in the absence of S phase cyclins DNA replication

occurs upon activation of the mitotic cyclins Clb 1, 2, 3, and 4. Thus, by all in vivo measures, initiation of DNA replication is defined by the activation of the Clb/Cdk1p kinases. However, the substrates that are phosphorylated in an event that causes initiation are completely unknown.

In addition to initiating DNA replication in G1, the Clb/Cdk1p kinases are thought to prevent spurious initiation (Dahmann *et al.*, 1995). Transient expression of Sic1p in G2 renders the origins of replication competent for initiation without an intervening mitosis. Subsequent repression Sic1p and reactivation of the kinase allows cells to undergo exactly one more round of DNA replication, indicating that the Clbp/Cdk1p kinases are the master molecules suppressing initiation until the cell cycle has been completed (Dahmann *et al.*, 1995). The priming of origins is not possible in the presence of Clbp/Cdk1p, but firing of these primed origins depends on it. The substrates which are phosphorylated by Clb/Cdk1p in a mechanism that prevents spurious replication are also unknown.

Since substrates in initiation have not been definitively identified, there is no event in the initiation of DNA replication that has been directly shown to be controlled by the action of the Clb/Cdkps. However, a number of steps in initiation exhibit cell cycle specific behavior, and a cell cycle pattern in turn suggests potential substrates because the oscillations of the cell cycle are governed by the reciprocal oscillations of the Clns (G1 cyclins) and the Clbs (S, G2 and M phase cyclins) (Amon *et al.*, 1993; Amon *et al.*, 1994). Phosphorylation of substrates may control replication in a number of ways: phosphorylation may cause the import or export of DNA replication proteins from the nucleus, phosphorylation may enhance or block the association of replication proteins in replication complexes, phosphorylation may trigger changes in conformation and activation or inactivation of activities of initiation proteins, and phosphorylation may target initiation proteins for degradation.

It has been shown that origins are licensed only during G1 in a normal cell cycle, and that after initiation ORC and Cdc6p are competent for the association with origin DNA, but Mcm4p, Mcm7p and Cdc45p are not (Aparicio *et al.*, 1997; Piatti *et al.*, 1996; Tanaka *et al.*, 1997). It is not clear what blocks reassociation of the latter proteins. Cytoplasmic localization of select Mcms may be directed by Clb/Cdk1p phosphorylation, and this in turn may be sufficient to account for the observation that the Mcms do not load onto origins in G2. However, certain *cdc6* mutants which overreplicate retain the Mcms on the chromatin, suggesting that inhibition of the association of the Mcms with Cdc6p is indeed one of the mechanisms by which initiation is regulated (Liang and Stillman, 1997). Destruction of a required protein could also account for lack of reinitiation. Quite a number of initiation proteins are periodically transcribed but corresponding protein levels are constant. Two key exceptions to this are Cdc6p and Dbf4p, which are known to be stable in G1 and unstable in S, G2 and early M phases (Piatti *et al.*, 1995, Dixon, Yang and Campbell, unpublished). The cell cycle dependent instability of Cdc6p and Dbf4p suggest that these proteins may be degraded in a Clbp/Cdkp dependent manner.

We are interested in characterizing the role of the Clbp/Cdkps in the triggering of initiation and the prevention of reinitiation. We have previously shown that Cdc6p both associates with and is phosphorylated by Clb/Cdk1p (Elsasser *et al.*, 1996). Our analyses to date suggest that while phosphorylation of Cdc6p in vivo has consequences that might be sufficient to regulate once and only once replication, there must be additional substrates that are targeted in both initiation and suppression of reinitiation, since mutation of Cdc6 phosphorylation sites is not sufficient to cause overreplication. Herein we describe the regulation of Cdc6p stability by Clb/Cdk1p.

MATERIALS AND METHODS

Media

The following abbreviations are used for yeast media: YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose), YPR (1% Bacto-yeast extract, 2% Bacto-peptone, 2% raffinose, YPG (1% Bacto-yeast extract, 2% Bacto-peptone, 2% galactose), SD ura- (2% dextrose, 7.5% yeast nitrogen base, 0.5% casamino acids, 20 mg/l adenine sulfate, 20 mg/l tryptophan), SR ura- (2% raffinose, 7.5% yeast nitrogen base, 0.5% casamino acids, 20 mg/l adenine sulfate, 20 mg/l L-tryptophan), SD leu- (2% dextrose, 7.5% yeast nitrogen base, 0.6 g/l CSM-his-leu-trp-ura (Bio101, Vista, CA), 20 mg/l histidine, 20 mg/l tryptophan, 20 mg/l uracil).

Strains

The strains used in this include **BJ2168** (*a prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52),* **BJ5459** (*a ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*) (Yeast Genetic Stock Center), **RJD635** (*a ura3 leu2 trp1 pep4::TRP1 cdc28::CDC28-HA::TRP1 bar1::LEU2*) (Elsasser *et al.*, 1996), **RJD885** (*a ura3 leu2 trp1 cln1::URA3 cln2::LEU2 cln3::URA3 leu2::GAL-CLN3::LEU2 pep4::TRP1 cdc28::CDC28-HA::HIS3*) (Verma *et al.*, 1997a), **RJD893** (*a cdc4 ura3 leu2 trp1 cln1::URA3 cln2::LEU2 cln3::URA3 leu2::GAL-CLN3::LEU2 pep4::TRP1 cdc28::CDC28-HA::HIS3*), **L40** (*a his3Δ200 trp1-901 leu2-3,112 ade2 lys2-801 URA3::(lexA OP)$_8$-LacZ LYS2::(lexA OP)$_4$-HIS3*) (Hollenberg *et al.*, 1995).

Plasmids

Preparation of vectors with the *GAL1,10* promoter (all constructs with *GAL* promoter). Yeast and Bluescript (Stratagene) plasmids bearing the *GAL* promoter flanked by SacI and BamHI sites was prepared as follows: pBM150 (Johnston and Davis, 1984) was cut with EcoRI, and an oligonucleotide cassette composed of SE25 and SE26 (Table 1) was cloned in. The oligocassette used has ends that anneal to the EcoRI ends but are designed such that the EcoRI sites are not regenerated, and the double-stranded region of the cassette contains a BamHI site. The *GAL* promoter was excised with SacI and BamHI, and cloned into the same sites in pBluescript SK(-), resulting in pELS42, and into the same sites in the yeast integrating vector with a *URA3* selectable marker pRS306 (Sikorski and Hieter, 1989), resulting in pELS89.

Introduction of a multiple myc tag at the C-terminus of *CDC6*. A Bluescript-based vector containing the coding region for a multiple myc epitope tag was generated by ligation of a cassette composed of oligos SE53 and SE54 (Table 1) and cloning these ladders into the SpeI site of pBluescript SK(-). A construct containing seven head to tail myc tags, pELS47, was selected for preparation of poly-myc-tagged *CDC6*. A fragment encoding residues 392 to 513 of *CDC6* (from the StyI site to the last residue) fused to a multiple myc tag was generated by recombinant PCR (Higuchi, 1990) using oligos SE61 through SE64 and templates pELS47 and pELS20 (Elsasser *et al.*, 1996). The resulting PCR product was cloned into the SacI and SpeI sites of pBluescript SK(-) generating pELS55. Sequencing of the PCR product revealed that two extra myc tags had been created during the course of the PCR reaction.

Preparation of yeast vectors expressing small N-terminal deletions of *CDC6* (Figure 1). pRS315-based vectors (*ARS1, CEN4, LEU2*) (Sikorski and

Hieter, 1989) for the expression of *CDC6* alleles with small deletions in the N terminus were assembled from three fragments: the *GAL* promoter, N-terminal fragments (from residue 1 to 48) bearing various mutations, and a fragment (from residue 47 to 513) bearing the C-terminus of *CDC6* fused the 9xMyc epitope tag. Mutagenesis of the N-terminus of *CDC6* was accomplished as follows: the region of *CDC6* encoding the first 48 residues of the protein was excised from pKTU6 (Elsasser *et al.*, 1996) with BamHI and PstI and cloned into the same sites in pBluescript SK(-), generating pELS12. (The sequence near the start of the open reading frame is GGATCCCATATG, where the start codon is underlined.) pELS12 was transformed into CJ236 (*dut⁻ ung⁻*), and single stranded DNA was prepared from this strain. Deletions within the C-terminal 46 amino acids were prepared with the oligonucleotide primers indicated in Table 1 using the Muta-Gene Mutagenesis Kit (BioRad, Hercules, CA) yielding pELS32 (Δ3-16), pELS33 (Δ13-26), pELS34 (Δ23-26), and pELS52 (Δ33-46). The *GAL* promoter was cloned in front of the mutated N-termini by inserting the SacI-BamHI fragment derived from pELS42 (above), generating plasmids pELS64 (WT), pELS68 (Δ3-16), pELS65 (Δ13-26), pELS69 (Δ23-26), and pELS66 (Δ33-46). A Bluescript-based construct bearing the *CDC6* open reading frame (ORF) from residue 47 to 513 fused in frame with the 9xMyc tag was generated by excising the natural C terminus with StyI and SpeI from pELS51, a bacterial vector bearing the *CDC6* open reading frame from residues 47 to 513 followed by 300 bases of the natural 3' untranslated region (UTR), and cloning in the epitope-tagged C-terminus from pELS55, resulting in pELS60. pELS60 was cut with PstI and HindIII, excising a fragment containing residues 47 to 513 of *CDC6* fused to the 9xMyc tag, followed by the natural 3' UTR and cloned into pRS315, resulting in pELS63. Fragments bearing the *GAL* promoter followed by the N-terminal amino acids (from pELS64 through

pELS69, described above) were cloned into pELS63, generating pELS71 (WT), pELS72 (Δ3-16), pELS73 (Δ13-26), pELS74 (Δ23-26), and pELS75 (Δ33-46).

Preparation of Bluescript-based vectors used for the in vitro ubiquitination assays. Mutation of the nuclear localization signal of *CDC6* was accomplished in a manner similar to that used to create the small N-terminal deletions, using the mutagenic primers indicated in Tables 1+2, resulting in pELS86. Bluescript-based constructs bearing the entire *CDC6* ORF were generated by cloning the PstI-HindIII fragment from pELS60 (containing the *CDC6* ORF from residue 47 to 513 fused to the 9xMyc tag, followed by the natural 3'UTR) into the vectors containing the wildtype and mutant N termini of *CDC6* (pELS12, pELS32, pELS33, pELS34, pELS52 and pELS86), resulting in plasmids pELS91, pELS92, pELS93, pELS94, pELS95, and pELS105, respectively.

Mutation of the Cdk consensus phosphorylation sites in *CDC6* was accomplished by transforming pELS91 into strain CJ236, producing single stranded DNA, and generating mutations using the Muta-Gene Mutagenesis Kit and the primers shown in Tables 1 and 2. For alleles with mutations at several sites, multiple oligos were used in a single reaction. Mutagenesis using several oligos at once produced anywhere from 10 to 90 percent desired products. The complete set of mutants is listed in Table 3.

An allele of *CDC6* lacking residues 8 through 46 was generated by swapping the NdeI-PstI fragment of pELS91 with a cassette comprised of oligos SE74 and SE75.

Plasmids with N-terminal glutathione-S-transferase tags. For the experiment shown in Figure 8, the plasmids YGpKTU6 (bearing wild type Cdc6p) and YGpME6 (bearing Cdc6p mutated at lysine 114 to glutamate) were used (Elsasser *et al.*, 1996).

Preparation of integrating yeast vectors used for the stability assays in Figure 3. BamHI-HindIII fragments from the Bluescript-based vectors containing the entire *CDC6* ORF, the C-terminal 9xMyc tag, and the natural 3'UTR (the same set that was used for the in vitro ubiquitination experiments) were cloned into pELS89 (pRS306 with the *GAL* promoter). For integration, vectors were linearized at the EcoRV site within the*URA3* marker.

Preparation of vectors used in the two hybrid assays. Vectors with the wild type and mutant *CDC6* ORFs fused at the C-treminus of the LexA DNA binding domain were prepared by amplifying sequences from the Bluescript constructs bearing the complete ORF (see Table 3) with 5' oligos SE128, SE129, SE130, and SE131, and 3' oligos SE132 and SE133 (see Table 4) and cloning into BTM116. The assortment of oligos used at the 5' end were required to amplify mutants with engineered sequence differences at the N-terminus. Inserts generated with SE132 were subcloned much more efficiently than inserts generated with SE133. The two oligos are exactly the same at the 3' end, which anneals to the last nine natural codons of *CDC6*. Following this, SE132 encodes a BamHI site and SE133 encodes a SalI site. BTM116 possesses a BamHI site followed directly by a SalI site in the polylinker between the LexA coding region and the transcriptional termination signals. In the cases where SE132 was used to generate inserts, the BamHI site is maintained after the stop codons. In the cases where SE133 was used, this site is removed.

Cdk1 binding

Strain BJ2168 was transformed with pELS71 through pELS75. 200 ml cultures were grown to mid log phase in SR-ura (2% raffinose, 0.5% casamino acids, 1.5% yeast

nitrogen base, and 20 mg/l each adenine and tryptophan). Expression of wild type and mutant *CDC6* was induced by adding solid galactose to 2%. Cells were harvested after four hours, washed and resuspended in High Salt Buffer (Elsasser *et al.*, 1996) at a concentration of 300 mg wet cells per ml, and lysed by beadbeating (Biospec Mini Beadbeater, Type BX-4), four times at 5000 rpm for 50 s each time, in the presence of 0.5mm Zir/Silica beads (Product # 11079105Z). The resulting extracts were diluted to 20 mg/ml, and small aliquots were removed and mixed with equal volumes of 4X SDS-PAGE loading buffer. 4 mg of the remaining extract was mixed with 2μg of 9E10 antibody and 40 ml of 100 μg/ml Protein A Sepharose slurry and incubated with mixing at 4°C for 90 minutes. Immunoprecipitated protein was recovered and beads were washed thoroughly. 20 ml of 4X SDS-PAGE loading buffer was added to the immunoprecipitates.

Stability assay

Degradation in synchronized cells. The following experiment was carried out at 30°C. 10ml cultures were grown overnight to early log phase (~ 2 x 10^6 cells/ml). Galactose was added at a final concentration of 2% to induce *CDC6* transcription, and cells were incubated for 2 h. Nocodazole was then added to a final concentration of 15 μg/ml, and cells were incubated for an additional 3 h. *CDC6* transcription was repressed by the addition of glucose to 2%. 0.75 ml aliquots of cells were removed at various times after the addition of glucose and added to an equal volume of ice cold 2X yeast stop mix (300 mM NaCl, 100 mM NaF, 2mM NaN_3, 20 mM EDTA). Cells were recovered by two centrifugations, and resuspended in 200 μl Lysis Buffer (50 mM NaOH, 2% SDS, 5% β-mercaptoethanol, 10% glycerol). After vortexing, samples boiled for five minutes and titrated with 1N HCl to a pH of 7. Debris was removed by centrifugation at 14,000 rpm for 5 min. The concentration of the resulting extract was ~4 mg/ml as determined by A_{280}, A_{260} readings. 150μl of the clear supernatants were transferred to a clean tubes and mixed

with 1μl bromophenol blue. 10μl of cleared extract was electrophoresed on 7.5% SDS-PAGE gels and blotted to Hybond nitrocellulose membranes (Amersham). Blots were incubated with 1:1000 dilution of 9E10 ascites (Santa Cruz Biotechnology), a 1:2000 dilution of goat-anti-mouse horseradish peroxide conjugate (BioRad), and developed with ECL reagents (Amersham).

Degradation in asynchronous cells. Procedure was the same as for synchronous cells, but cells were grown exponentially to a density of (1 x 10^7 cells/ml) before induction.

In Vitro Transcription and Translation

DNA templates for transcription of myc tagged alleles of Cdc6p were prepared by PCR amplification of *CDC6* sequences from Bluescript-based plasmids listed in Table 3. The 5' primers were designed to introduce the T7 promoter upstream of the *CDC6* coding region (SE108, SE134, SE135 and SE136) and the 3' primer SE37, a sequence from the *CDC6* 3' UTR. These primers are listed in Table 1. In all cases the 5' primers anneal to the first 27 nucleotides, which are the same as wild type in most cases, but different for the alleles with a mutation at the first cdk consensus site (threonine 7 → alanine), and for the deletions Δ3-16 and Δ8-48. Amplification was carried out using 1 ng of plasmid DNA and 0.2μM primers. DNA concentrations were determined by densitometry using an Alpha Innotech Gel Documentation System. A DNA template for the transcription of *SIC1* was prepared as described (Verma *et al.*, 1997a). A DNA template for the transcription of untagged *CDC6* was produced by amplifying the open reading frame from the plasmids listed in Table 3 with primers SE102 and SE103. PCR products were purified using the Qiagen PCR Purification Kit. Transcription was carried out in a volume of 100 μl by incubating 1 μg DNA in the presence of 1 mM NTPs, 100 U RNAse Inhibitor (Boehringer

Mannheim Biochemicals, product # 799025, 1U inhibits 50% of 5 ng RNAse A), 2U T7 RNA polymerase and accompanying buffer (Boehringer Mannheim Biochemicals) for 75 min at 37°C. 3 ml of RNA was used directly in 20 ml Wheat Germ In Vitro Translation reactions (Promega) as per manufacturer's instructions for both hot and cold translations.

Preparation of Fractionated Extracts for Ubiquitination

Preparation of DEAE fractionated cell extracts was carried out as previously described (Verma *et al.*, 1997a), and as follows: Strain RJD885 was used for wild type cell extract, and strain RJD893 was used for *cdc4* extracts. Cells were grown in 1L YPG (1% yeast extract, 2% peptone, 2% galactose) to a density of $1x10^7$ cells/ml, recovered by centrifuging for 15 min at 5000 rpm, resuspended in the same volume of YPD and incubated for 5.5 h at 24°C to deplete cells of Cln2p and arrest cells in G1. Cells were recovered by centrifugation (as above), washed with 200 ml ice-cold water, collected in one 50 ml Faclon tube, and extruded as into liquid N_2 by piercing the bottom of the tube with a 18.5 gauge needle and forcing the cell paste out with a plunger from a 60 ml syringe. A typical yield was 5 g/l. Frozen cells were ground under liquid nitrogen with a mortar and pestle for 10 min, and frozen powder was thawed at a concentration of 2 g/ml in Buffer A (30 mM HEPES, pH 7.2, 100 mM potassium acetate, 1mM EDTA, 1mM $MgCl_2$, 10% glycerol, 2mM DTT, 1mM PMSF) fortified with an additional 200 mM potassium acetate. Extract was incubated on ice for 15 min and centrifuged for 15 minutes at 50,000 rpm at 4° in a TLA 100.3 rotor. The supernatant was adjusted to 2mM magnesium acetate, 2mM $CaCl_2$, 10 mg/ml RNAse, 20 mg/ml DNAse, 0.9 U/ml hexokinase, and 50 mM glucose, and incubated at 16°C for 30 min, and dialyzed against Buffer B (25 mM HEPES, pH 7.6 , 25 mM NaCl). After sedimenting insoluble protein, the dialysate was applied to a DEAE-Spharose-FF column (1.5 cm in width) at a

concentration of 5 mg/ml of bed volume. The column was washed successively with 2 vol of Buffer B, Buffer B plus 25 mM NaCl, Buffer B plus 225 mM NaCl, and Buffer B plus 500 mM NaCl. Proteins eluting at 250 mM NaCl were concentrated in a Centriprep-10 (Amicon) to a concentration of 25 mg/ml (10-20 fold). Protein was flash frozen in 50 µl aliquots. This fraction contains Uba1p (E1), Cdc4p, Cdc53p, and Skp1p (E3 complex), but lacks Cdc34p (E2), all cyclin/cdks, and ubiquitin.

Purification of Cdc34p

Cdc34p was purified as previously described (Banerjee *et al.*, 1993). For the experiments in Figures 4 and 5, Cdc34p purified to homogeneity was used. For the experiment in Figure 6, Cdc34p purified through the DEAE step (25% pure) was used.

Expression of Proteins in Insect Cells

Baculoviruses used for the expression of proteins in insect cells were as follows: vectors for the expression of GST-Cdk1^{HA}p, Cln2p, Clb5p and Cdc4p were the same as those previously described (Feldman *et al.*, 1997). For the production of insect cell lysates containing Cln2p/Cdk1p and Clb5p/Cdk1p, SF9 insect cells were coinfected with the relevant viruses at an MOI of 10. At 42 hours post infection, cells were recovered by centrifugation and resuspended 0.1 vol in ice cold Insect Cell Lysis Buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 0.1% Triton-X100, 5 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, 1 µg/ml leupeptin and pepstatin, 50 mM NaF, and 50 mM β-glycerophosphate). Cells were briefly sonicated to ensure lysis. The lysate was cleared by centrifugation for 15 min at 13,000g, brought to a glycerol concentration of 10%, and frozen in aliquots in liquid nitrogen.

Phosphorylation Assays

Untagged *CDC6* was amplified and [^{35}S]-labeled Cdc6p was produced by in vitro translation as described above. 2 µl of translation product was mixed with 4 µl insect cell lysate containing GST-Cdk1-HAp and Clb5p, 2 µl of 10X reaction salts and protease inhibitors (50 mM magnesium acetate, 10 mM DTT, 5 mM PMSF, and 100 µg/ml each leupeptin and pepstatin), 2µl of an ATP regenerating system (10 mM ATP, 350 mM creatine phosphate, 20 mM HEPES pH 7.2, 10 mM magnesium acetate, and 0.5 mg/ml creatine kinase), and 10 µl distilled water. Half of the reaction was quenched immediately with SDS-PAGE loading buffer, and the other half of the reaction was incubated for 60 min at 25°C. The reaction was stopped by the addition of SDS-PAGE loading buffer.

Ubiquitination Assays

Ubiquitination reactions were carried out by combining 1 µl of in vitro translation mix containing substrate (Cdc6-Myc9p or ^{35}S labeled Cdc6p or Sic1p, 1 µl of insect cell lysate containing kinase, 4 µl of DEAE fractionated yeast extract (as described above), 0.2 µg Cdc34p (as described), 1 µl of ATP regenerating system (10 mM ATP, 350 mM creatine phosphate, 20 mM HEPES pH 7.2, 10 mM magnesium acetate, and 0.5 mg/ml creatine kinase), 1 µl of 10X reaction salts and protease inhibitors (50 mM magnesium acetate, 10 mM DTT, 5 mM PMSF, and 100 µg/ml each leupeptin and pepstatin), and 0.5 µl of 20 mg/ml bovine ubiquitin (Sigma). Reactions were brought to a volume of 10 µl with 20 mM HEPES pH 7.2, 100 mM potassium acetate. Reactions were mixed and incubated at 25°C for 1 h. Translation produced some high molecular weight products, which have been previously observed (Verma *et al.*, 1997a), and are thought to be protein-RNA adducts resulting from inefficient translation termination. Where indicated, translation products were treated with 20 µg/ml RNAse A at 37°C for 15 min, which served to remove a small

amount of high molecular weight background and improved the sensitivity of the ubiquitination assay.

Two Hybrid Assay

Strains for the two hybrid assay were created by transforming L40 with pairs of plasmids. All strains contained the pACT-CDC4 plasmid (bearing *CDC4* fused to the activation domain of *GAL4* (Drury *et al.*, 1997) and one of the following: BTM116, which carries the lexA DNA binding domain, pBTM-CDC6 (Drury *et al.*, 1997), and the plasmids shown in Table 4.

The two hybrid assay was carried out essentially as described in the Clonetech manual: saturated cultures of cells were aliqoutted onto solid selective medium and incubated for 24 h at 30°C. 75 mm paper filters (VWR product # 28321-055) were used to lift cells from the plates. Filters were frozen for ten seconds in liquid nitrogen, colony side up, then thawed at room temperature. Two clean filters were placed in a petri dish saturated with Z buffer. Thawed filters were placed on top of filters saturated with Z buffer (100 mM sodium phosphate pH 7.0, 10 mM KCl, 10 mM $MgSO_4$, 40 mM β-mercaptoethanol, 0.3 mg/ml X-gal), and incubated at 30°C until a blue color developed in the positive control (about 75 min). Filters were then dried with a heat gun, which served to stop the reaction.

RESULTS

The N-terminus of Cdc6p Contains Signals Important for Regulated Destruction of Cdc6p

The interaction of Cdc6p with Cdk1 could exist only for targeting of Cdk1p to its substrates in the replication apparatus or for phosphorylation of Cdc6p or both. In order to investigate further the nature of the Cdc6p/Cdk1p interaction, we have carried out mutagenesis within subdomains of the Cdk1 binding region of Cdc6p and have changed the serines or threonines in the six Cdk1 consensus phosphorylation sites in Cdc6p to alanine. We have analyzed individual mutations and various combinations of mutations. Our first insight into one role of the Cdc6p/Cdk1p interaction came from analysis of a series of deletions within the Cdc6p N-terminus, Δ8-48, Δ3-16, Δ13-26, Δ23-36 and Δ33-46. Genes containing these deletions were tagged at the C-terminus with nine copies of the Myc epitope. (The *CDC6-Myc9* construct complements a *cdc6* null.) The tagged mutant genes were then expressed under control of the *GAL1,10* promoter in yeast. In the first set of experiments, extracts of cells expressing each of the mutants were immunoprecipitated with anti-myc 9E10 monoclonal antibody, and the immunoprecipitates were probed for the presence of Cdk1p by Western blotting. As shown in Figure 1, Cdk1p was present in wildtype Cdc6-Myc9p immunoprecipitates, but it was not associated with any of the mutant proteins. Control Western blots indicated that each mutant Cdc6p was expressed at least at levels as high as wildtype (Figure 1). We conclude that the absence of Cdk1p was due to failure of mutant Cdc6p to bind Cdk1p. Failure to interact could either be due to altered structure of the deleted proteins or to deletions of signals important for interaction. We prefer the latter interpretation since all mutants do support growth in the absence of wildtype Cdc6p, showing that at least the portion of the protein required for replication is correctly matured.

We noticed in the experiment shown in Figure 1, that Cdc6Δ23-36p appeared to accumulate to higher steady state levels than did wildtype (or any of the other mutants). Since one of the interesting aspects of regulation of Cdc6p is the fact Cdc6p, unlike most other DNA replication proteins, has been shown to be unstable in vivo after the G1/S transition, we next asked if the accumulation of Cdc6Δ23-36p was due to stabilization of the Cdc6p. Cells expressing the Myc-tagged wildtype and mutant proteins under the control of the *GAL1,10* promoter were grown in galactose, synchronized in G2 by the addition of nocodazole, and then shifted to glucose medium to repress expression of Cdc6p. The level of Cdc6p remaining during subsequent incubation in glucose medium Cdc6p was then followed by protein blotting. Two mutants, Cdc6Δ8-48p and Cdc6 Δ23-36p, were indeed stabilized relative to wildtype (Figure 2). These results show that there are motifs within the 48 N-terminal amino acids that are important for determining the stability of Cdc6p in the cells cycle, in agreement with recent results of others (Drury *et al.*, 1997). Furthermore, we show that deletion of amino acids 23 to 36 is a sufficient condition to cause stabilization. These results do not say that the only signals important for stability of Cdc6p within the N-terminus reside between amino acids 23 and 36, however. Although Cdc6Δ3-16p, Cdc6Δ13-26p, and Cdc6 Δ 33-46p were unstable, each of these mutants might contain signals that alone do not lead to stabilization, but when combined with other signals do so. In particular, amino acids 3-16, 13-26, and 33-46 contain Cdk consensus phosphorylation sites, and amino acids 17-46 have previously been proposed to contain binding sites for the Cdc4p protein, required for destruction of Cdc6p in vivo (Drury *et al.*, 1997; Holmes and Solomon, 1996; Piatti *et al.*, 1996). Further analysis of all of these sites is described below.

Mutation of the Nuclear Localization Signal Leads to Decreased Turnover of Cdc6p

Because of the marked increase in stability of Cdc6Δ23-36p, we have investigated this region more carefully. There are at least two motifs between amino acids 23 and 36 that might be important for regulated destruction of Cdc6p. First, a conserved nuclear localization signal (NLS), ^{27}PLKRKKL^{33}, has been shown to be functional by the failure of K29T and K29R mutations to direct β-galactosidase to the yeast nucleus (Jong *et al.*, 1996). Second, S23 falls in a consensus Cdk phosphorylation motif. We first investigated the effect of mutation of the NLS alone on Cdc6p stability. Amino acids 29-32, KRKK, were changed to AAAA and the mutant gene was tagged with the myc epitopes and expressed from the *GAL* promoter. Using the glucose shut-off strategy already described, we found that this four amino acid mutation does lead to stabilization of Cdc6p (Figure 2). The extent of stabilization due to changing the NLS is equivalent to the effect generated by deleting residues 23 through 36, suggesting that the nuclear localization signal is the key, non-redundant motif in this region contributing to the stability of Cdc6p. (Using fusions to green fluorescent protein, we have shown that the KRKK-to-AAAA mutant fails to concentrate Cdc6p in the nucleus (Luo, Elsasser and Campbell, unpublished). These results suggest that destruction of Cdc6p takes place in the nucleus.

Specific Phosphorylation Sites are Required for Cdc6p Turnover In Vivo

We next tested the contribution of the six sites in Cdc6p most likely to be phosphorylated by Cdk1, Cdk consensus phosphorylation sites, to the stability of Cdc6p (Holmes and Solomon, 1996). The serines and threonines at T^{7}TPK, T^{23}PPR, S^{43}PEK, T^{134}PR, S^{354}PTR, and S^{372}PVK were changed to alanine, singly and in combination. These sites are referred to as cdk 1 through cdk 6. Mutants were tagged with the Myc9 epitope and expressed under *GAL* control and stability of the mutant proteins analyzed as

described in Figs. 1 and 2. Mutation of the single cdk 1, cdk 2, cdk 3, cdk 4, or cdk 5 sites caused no change in stability compared to wildtype (Figure 3A). However, mutation of cdk 6 led to significant stabilization. This is interesting and unexpected since S372 lies in a C-terminal region dispensable for the tight binding to Clb/Cdk1p we had reported previously and could not account for the stabilization observed in Cdc6Δ8-46p. Nevertheless, the C-terminal portion of Cdc6p is phosphorylated by Clb5p/Cdk1p in vitro (Figure 3B). Since we had observed previously that the N-terminus is strongly phosphorylated in vitro by Cdk1p (Elsasser *et al.*, 1996) and that deletions that remove the N-terminal consensus sites are stabilized, we tested the effects of simultaneous mutation of multiple cdk sites in the N-terminus. Indeed, the cdk 1-3 triple mutant was stabilized (Figure 3). The same level of stability observed in the triple mutant was also obtained in the cdk 1+3 double mutant, whereas cdk 2+3 showed a modest increase in stability, but cdk 1+2 showed no difference from wildtype. Thus, phosphorylation sites in the N-terminus are also important for destruction of Cdc6p in vivo. These results taken together suggest that phosphorylation at both the N- and C-termini influence Cdc6p stability. The two sets of sites may have redundant or additive roles in vivo, or there may be several steps in the regulation of degradation in vivo which are each dependent on phosphorylation at specific cdk sites.

Roles of Phosphorylation in Degradation of Cdc6p

There are a number of models that might explain the requirement for phosphorylation in the degradation of Cdc6p. Since degradation appears to take place in the nucleus, phosphorylation of Cdc6 may be required for nuclear localization or association with chromatin. In this case, the role of phosphorylation is indirect. Alternatively, phosphorylation might be directly required for degradation. It has been proposed that Cdc6p is targeted to the 26S proteasome by ubiquitination through the SCF-

Cdc4 pathway, since Cdc6p degradation is inhibited in *cdc53*, *cdc34*, and *cdc4* mutants, and since Cdc6p appears to interact with Cdc4p in two hybrid assays (Drury *et al.*, 1997). However, neither ubiquitination nor phosphorylation of Cdc6p has been shown to be involved in regulating Cdc6p stability. Since we observed that mutation of the NLS generated a stabilized allele of Cdc6p, we considered it possible that the cdk 1-3 and cdk 1-6 mutant proteins were no longer nuclear. In work to be described elsewhere, we have shown that these mutant proteins show wildtype subcellular localization (Luo, Elsasser, and Campbell, unpublished). In order to explicitly differentiate between direct and indirect effects, such as nuclear localization or chromatin binding, we analyzed ubiquitination of Cdc6p in an in vitro reaction that has been shown to faithfully mimic in vivo events (Feldman *et al.*, 1997; Skowyra *et al.*, 1997; Verma *et al.*, 1997b).

Ubiquitination of substrates in vitro generally requires the presence of ubiquitin, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3), the last of which is typically substrate specific. Cdc34p is an E2-type enzyme, and together, Cdc34p, and three additional proteins, Cdc4p, Cdc53p and Skp1(an E3 known as SCF-Cdc4) target the Cdk inhibitor Sic1p for ubiquitination and degradation at the G1/S phase transition (Schwob *et al.*, 1994). This reaction has recently been recapitulated in vitro (Feldman *et al.*, 1997; Skowyra *et al.*, 1997; Verma *et al.*, 1997b), and we have adapted this system to the study of Cdc6p. Since there is strong evidence that Cdc6p is degraded at the G1/S transition, we used extracts prepared from cells arrested in G1 by turning off the Cln kinases (Deshaies *et al.*, 1995). Since the Cln/Cdks are required for activation of the Clb/Cdks, these extracts are devoid of all Cdk phosphorylating activities. They are thus ideal for testing both whether Cdc6p is ubiquitinated and if that ubiquitination is dependent on phosphorylation by Clb/Cdks.

The G1-arrested extract has been shown to contain the ubiquitin activating enzyme Uba1p (E1), as well as at least one E3, namely SCF-Cdc4 (Verma *et al.*, 1997a; Verma *et*

al., 1997b). Cdc34p (E2) and ubiquitin are added to the extract to reconstitute the complete ubiquitinating system. For our experiments, Sic1p was the positive control for the ubiquitination reactions (Verma *et al.*, 1997a; Verma *et al.*, 1997b). As shown in Figure 4, lanes 3 and 6, when in vitro translated ^{35}S-Sic1p and Cdc6p are added to this reconstituted system, Cdc6p, like Sic1p, is converted to more slowly migrating conjugates, which we attribute to ubiquitination based on the requirements for the reaction demonstrated below. The conclusion that the slowly migrating bands in Figure 4, lane 6 are species of Cdc6p is based on the fact that the bands are visualized by Western blotting with an antibody against the Cdc6-Myc9p. Importantly, neither Sic1p nor Cdc6p is ubiquitinated in the absence of added Cdk kinase (Figure 4, lanes 2 and 4). However, there is a significant difference between the kinases required for the two different substrates. Sic1p ubiquitination is preferentially stimulated by Clnp/Cdk1p (Figure 4, compare lanes 2 and 3) as reported previously (Verma *et al.*, 1997b), although Sic1p appears to be a substrate for the Clb5/Cdk1 (Figure 4, lane 3). In contrast, Cdc6p ubiquitination is stimulated by Clb5p/Cdk1p but almost not at all by Cln2p/Cdk1p (Figure 4, compare lanes 5 and 6). (We cannot resolve phosphorylated and unphosphorylated forms of Cdc6p tagged with the Myc9 epitope). This difference in cyclin requirement reflects the expected specificity of the reactions, since Sic1p is by definition degraded before activation of Clb/Cdks, and Cdc6p interacts preferentially with Clb/Cdks and not Cln/Cdks (Elsasser *et al.*, 1996). We propose that phosphorylation of Cdc6p, probably by a cyclinB/Cdk1 kinase, stimulates targeting of Cdc6p to the ubiquitin apparatus.

If true, then the requirement for Clb/Cdk1p for Cdc6p degradation raises the question of whether Cdc6p is stable in *cdc4*, *cdc34* and *cdc53* mutants because the gene products are directly required for Cdc6p degradation or whether it is an indirect effect, since their function is necessary for Sic1p degradation and hence activation of the Clb/Cdks. The in vitro system allows us to ask whether Cdc34p and Cdc4p are directly

required for ubiquitination of Cdc6p. As shown in Figure 4, lanes 7 and 8, if the recombinant Cdc34p is omitted from the reaction, the high molecular weight conjugates of Cdc6p are not observed. In Figure 5, we show that Cdc4p is also required for in vitro the reaction. In this experiment, extracts were prepared from both wildtype and *cdc4* cells arrested at the restrictive temperature. In vitro reactions were then carried out as in Figure 4. No slowly migrating species are obtained in the reaction using extract prepared from the *cdc4* cells (compare lanes 5 and 10, Figure 5). If insect cell lysate containing recombinant Cdc4p is added to the reaction containing the *cdc4* extracts, however, ubiquitination of Cdc6p is restored (Figure 5, compare lanes 10 and 12). We conclude that the slowly migrating species are ubiquitinated Cdc6p (or are modified by ubiquitin-like proteins). Furthermore, we propose that Cdc34p and Cdc4p are directly required for Cdc6p destruction and that Cdc4p is required for recognition of Cdc6p as a substrate for ubiquitination.

Mapping of the Determinants in Cdc6p Required for Ubiquitination In Vitro

Having demonstrated that Cdc6p could be ubiquitinated in the Clb5/Cdk1, Cdc34p, Cdc4p dependent in vitro reaction, we used this assay to evaluate whether or not the stability of the cdc6 mutants we described above was correlated with a lack of ubiquitination in vitro. In each case, the physiological significance of the in vitro ubiquitination reaction was controlled for by demonstrating dependence on Cdc34p (Figure 6). First, the Cdc6Δ8-46p was not ubiquitinated. If the only motif in this region necessary for stabilization were the NLS, then we would have expected this protein to be ubiquitinated in vitro, where all of the required components for ubiquitination had access to the Cdc6p. Instead, it appears that additional signals within these amino acids were important specifically for ubiquitination. Deletion of amino acids 3-16 or 13-26 did not appear to reduce ubiquitination relative to wild type. Deletion of amino acids 23-36, which

contains the NLS discussed above, did appear to reduce ubiquitination. Thus, the NLS is probably not the only signal even in this small region important for destruction. Confirming this interpretation, we observed that the specific NLS (KRKK to AAAA) mutant, which generated a stabilized allele of Cdc6p, was indeed ubiquitinated at a level equivalent to wild type (Figure 6, lanes labeled NLS). This supports the idea that the role of the NLS in degradation is simply to concentrate Cdc6p in the nucleus, rather than, for instance, supplying a lysine crucial for the attachment of ubiquitin. Finally, what we had not anticipated, because the mutant is as unstable as wildtype, was that deletion of amino acids 33 to 46 significantly lowered ubiquitination, a result repeated in many other experiments.

We next tested the phosphorylation site mutants for ubiquitination. As shown in Figure 6, mutations in individual cdk sites did not abolish ubiquitination of Cdc6p, even for the stabilized cdk 6 site. (Normal ubiquitination of the cdk1 mutant is observed in other experiments and the weak signal here probably reflects an inefficient in vitro translation reaction (data not shown)). Since the cdk 6 mutant is stabilized, it was surprising that the cdk 6 mutant was ubiquitinated, which suggests that either this mutant protein is protected from ubiquitination in vivo through interaction with another protein or, if it is ubiquitinated in vivo, that its degradation is blocked at a step downstream of ubiquitination.

While mutation of individual cdk sites did not affect in vitro ubiquitination, strong effects were seen in multiple mutants. Mutation of all three N-terminal cdk sites, cdk 1-3, abolished ubiquitination completely. No further reduction was seen when the additional sites, cdk1-6 were mutated. Interestingly, again, mutation of cdk 5 and 6, still allowed ubiquitination, though these produced stabilized Cdc6p in vivo (Figure 3). Double mutants in the N-terminal phosphorylation sites, at cdk sites 2 and 3 or 1 and 3 significantly reduced ubiquitination and increased stability (Figures 6 and 3). Thus, there is an inverse correlation between ability to be ubiquitinated and ability to be phosphorylated in the N-

terminal region. However, lack of phosphorylation of the C-terminal cdk sites does not prevent ubiquitination.

Two hybrid analysis of the interaction of Cdc6p with Cdc4p

Sine phosphorylation of substrate is not an obligate step in ubiquitination reactions in general, we wanted to test whether the observed failure of ubiquitination was a result of a failure in recognition by Cdc4p, a component of the ubiquitin E3 which is thought to confer substrate specificity. It has been shown that phosphorylation of Sic1p is required for recognition by Cdc4p (Feldman *et al.*, 1997). Although many types of experiments were attempted, we have not been able to show direct binding of Cdc6p to Cdc4p in yeast extracts. Therefore, we turned to the yeast two hybrid assay for protein interaction. The mutant alleles were cloned as LexA fusion proteins and their ability to interact with a *CDC4-GAL4* activation domain fusion protein assayed. The results are shown in Figure 7. In every case where ubiquitination was observed (Figure 6), Cdc4 binding was also observed. In one case, Δ33-46, ubiquitination was very weak and the binding to Cdc4 was weaker than the limit of detection in this two hybrid scheme. More interesting is the fact that the cdk 6 mutant, which is stable in vivo, was both ubiquitinated (Figure 6) and was observed to bind Cdc4p apparently as well as wildtype (data not shown). This result again suggests that phosphorylation of the C-terminal sites regulates a different part of the destruction pathway than phosphorylation of the N-terminal sequences.

A cdc6 Mutant that Fails to Bind Chromatin Productively is Hyperunstable

One possible explanation for the behavior of the cdk6 mutant is that it binds more tightly to chromatin and is somehow protected from degradation, even though it is ubiquitinated. To begin to test this idea, we needed to assess whether Cdc6p is degraded when bound to chromatin. We have recently generated an allele of *CDC6* mutated in the

Walker A box of the ATP binding sites and shown that this mutant cannot support DNA replication (Jong, Elsasser and Campbell, unpublished). The Walker A box mutant (K114E) fails to form a prereplicative origin footprint, and presumably has a defect in chromatin binding (Perkins and Diffley, 1998). This affords a way to ask if chromatin binding affects the stability of Cdc6p. The K114E mutant was tagged with the Myc9 epitope, expressed under *GAL* control, and the stability of the protein assessed as in Figure 2 and 3. As shown in Figure 8, the protein is less stable than the wildtype protein. This result supports the idea that binding to chromatin may affect the half-life of the Cdc6 protein. If so, the cdk 6 mutant may be stabilized through tighter binding to chromatin. However, an important caveat is that we have not shown that the degradation of the K114E mutant is dependent on Cdc4p.

Phenotypes of the Mutants

Each of the *GAL-CDC6-MYC9* mutants was transformed into a strain carrying the wildtype *CDC6* gene under the control of the repressible *MET* promoter (Piatti *et al.*, 1996). Transformants were then plated on medium containing methionine to repress the wildtype gene and ask if the mutants support growth. All mutants supported growth on galactose medium. Overproduction of wildtype Cdc6p leads to cells with elongated buds, and consistent with previous observations, Cdc6Δ8-48p does not give this phenotype (Elsasser *et al.*, 1996). Since we have pointed out that there are many regulatory signals within amino acids 8 to 48 (Cdc4 binding, Cdk1 binding, NLS, Cdk phosphorylation sites), we assessed the morphology of the whole array of mutants studied here to determine the motif most likely to contribute to the abnormal morphology. Of all the mutants, only the deletion mutants Δ3-16, Δ13-26, Δ23-36 and Δ33-46 show normal bud morphology. Of all defects, the only defect that all four of these mutants share is the inability to bind tightly to Cdk1p, suggesting that this is the primary defect leading to the elongated buds.

Interestingly, expression of the N-terminal 48 amino acids alone does not lead to hyperpolarization (data not shown), indicating that the Cdk1 binding region of Cdc6p only leads to bud hyperpolarization in the context of native Cdc6p.

We have sought conditions under which the effect of these mutations could be revealed in vivo. It has been shown that *cdc16-243* mutants rereplicate their DNA at the restrictive temperature. Since Cdc16p is thought to be part of the APC, a complex required for the degradation of cyclins at the metaphase to anaphase transition, it seemed possible that failure to degrade one or more replication proteins might account for the *cdc16* overreplication phenotype. If so, then introduction of the stabilized alleles of Cdc6p might exaggerate the overreplication. The *cdc6* mutants Δ8-48, cdk 1-3 and cdk1-6 were transformed into a *cdc16-243* strain. Transformants were grown on glucose and then tested for growth after induction on galactose plates. In this genetic background, cdk1-6 mutants showed a distinct dominant negative phenotype, killing the cells even at the permissive temperature. In contrast to the specific phosphorylation site mutants, the Cdc6Δ8-48p and cdk 1-3 did not kill the cells. Therefore, the result is not simply due to stabilization and shows that phosphorylation of both the N-terminal and the C-terminal residues effects more than just the stability of Cdc6p.

DISCUSSION

Requirements for Stability of Cdc6 In Vivo

Rapid degradation of Cdc6p homologs in other eukaryotes suggests that this instability has important implications for regulation of cell cycle progression. In this work we have explored the role of phosphorylation of Cdc6p in its regulated destruction during the cell cycle. By using an in vitro system that avoids ambiguities introduced due to in vivo compartmentalization, we have demonstrated that Cdc6p is ubiquitinated and that Cdc34p and Cdc4p, which are also required for Cdc6p degradation in vivo, are directly involved in ubiquitination of Cdc6p. Phosphorylation of the three N-terminal Cdk phosphorylation sites appears to be required for Cdc4p binding and for ubiquitination, suggesting that one role of phosphorylation is targeting to the ubiquitination machinery. We have also found that Clb/Cdks are more effective at targeting Cdc6p for ubiquitination than are Cln/Cdks. Phosphorylation of a fourth Cdk1 site at the C-terminal region of Cdc6p also stabilizes Cdc6p but is not required for Cdc4p binding or for ubiquitination, suggesting that ubiquitination is not sufficient to target Cdc6p to the proteasome. During the course of the study, we also found that nuclear localization is important for the destruction of Cdc6p. Since the NLS mutants are efficiently bound by Cdc4p in the two hybrid assay and are ubiquitinated, it does not appear that phosphorylation regulates the concentration of Cdc6p in the nucleus, and, supporting this interpretation, in studies to be reported elsewhere, we rule out that phosphorylation of the sites studied to date is required for nuclear localization of Cdc6p (Luo, Elsasser and Campbell, unpublished).

Since Cdc6p had been shown to be stable in cells with *cdc4, cdc53 and cdc34* mutations, and since Cdc6p had been shown to interact with Cdc4p in two hybrid assays, it was considered likely that Cdc6p is degraded through ubiquitination and targeting to the 26S proteasome. Studies of the fission yeast Cdc6p homolog Cdc18p have shown that

high molecular weight conjugates of Cdc18p can form in proteasome mutants, but there is some uncertainty as to which ubiquitin ligase mediates this process in vivo, since both pop1+ (a Cdc4p homolog) and sud1+ have been implicated in the degradation of Cdc18p (Kominami and Toda, 1997; Jallepalli *et al.*, 1998). However, no one had demonstrated that Cdc6p is in fact ubiquitinated in vivo or in vitro, leaving open the possibility that the effect of the *cdc4, cdc34 and cdc53* mutations is indirect. Therefore, it is important that we have shown that Cdc6p ubiquitination in vitro requires Cdc4p and Cdc34p.

The role of phosphorylation in regulating the stability of Cdc6p was not previously demonstrated. However, several other proteins have been shown to be targeted for degradation by phosphorylation: Sic1p, Far1p and the G1 cyclins. For these other G1 substrates of the 26S proteasome, phosphorylation at multiple sites is required for degradation, and this is also true for Cdc6p. Only a subset of the Cdk sites on Cdc6p are required for ubiquitination, as with Sic1p, and no single site is sufficient. As observed for Sic1p and Far1p, but not the G1 cyclins, phosphorylation of Cdc6p seems to be essential specifically for binding of Cdc4p, since there is a somewhat of a correlation between Cdc6p phosphorylation site mutants that cannot be ubiquitinated and those that fail to bind Cdc4p. That is, SCF-Cdc4 targets phosphorylated Cdc6p for destruction. However, there is a significant difference between the kinases involved in the targeting of Sic1p, Far1p and Cdc6p for destruction. Sic1p and Far1p are preferentially targeted to SCF-Cdc4 by Cln2p/Cdk1p, while Cdc6p is preferentially targeted by Clb5p/Cdk1p. Because activation of these kinases is temporally controlled, this allows for staggering of the degradation of Sic1p and Cdc6p. Thus, Cdc6p cannot be degraded (and the pre-replicative complex thereby inactivated) until Sic1p is removed and the Clb/kinases are able to execute initiation of replication.

The regulation of Cdc6p also differs from that of Sic1p in that Cdc4p binding, ubiquitination and instability do not strictly correlate. The Cdc6(cdk6) protein is stabilized

but is still ubiquitinated and binds Cdc4p as well as wildtype. To date, phosphorylation had only been demonstrated to regulate association of a degradation substrate with the SCF ubiquitination machinery. The cdk 6 allele of Cdc6p, however, is presumably stable either because this allele is not available to the ubiquitination machinery in vivo, or because it is ubiquitinated but not targeted to the proteasome for destruction. The pathway of delivery of ubiquitinated proteins to the proteasome is very poorly understood, and this *cdc6* mutant may offer some insight into that process.

Since Cdc6p must be nuclear in order for degradation to occur, it was possible that Cdc6p was only degraded when associated with chromatin. To investigate this, we determined the stability of the Cdc6K114Ep mutant that binds chromatin inefficiently (Perkins and Diffley, 1998). We found that the protein was even less stable than wildtype, suggesting that in yeast, that association of Cdc6p with the chromatin confers a stabilizing effect on Cdc6p. Conversely in *Xenopus*, it appears that only Cdc6p that is associated with the chromatin is degraded, and furthermore, degradation may be required for Mcm loading in this system (Hue and Newport, 1998). This is not as inconsistent with the observations in the *Xenopus* system as it sounds, however, since *Xenopus* Cdc6p that associates with chromatin after the window for initiation has closed is stable. In addition, the bulk of Cdc6p in *S. cerevisiae* is degraded after the G1/S transition, but in Xenopus, Cdc6p appears to be sequestered over the course of the cell cycle.

In Vivo Effect of Stabilizing Cdc6p

We have found no effect on growth due to the overproduction of any of the Cdc6 mutant proteins in a wild type background. Contrary to expectations from observations in *S. pombe*, overproduction of Cdc6p does not lead to overreplication in yeast. For such a key process such as high fidelity replication of the chromosome, it is not unexpected that there would be redundancy in the mechanism that prevents rereplication, and arguably the

fact that the overproduction of Cdc6p in *S pombe* causes overreplication is more surprising than the converse. In *S. cerevisiae,* other candidates for proteins that may be regulated in a mechanism that prevents initiation in G2 include the Mcms, which have been demonstrated to be compartmentalized over the course of the cell cycle, and Dbf4p, which is unstable after the G1/S transition (Dixon, Yang and Campbell, in preparation). In addition, the anaphase promoting complex appears to play a role in preventing aberrant initiation, as DNA overreplicates in *cdc16* mutants (Heichman and Roberts, 1996; Heichman and Roberts, 1998). This observation suggests that one or more DNA replication proteins may be targeted for degradation by the anaphase promoting complex, and although Cdc6p would seem a good candidate, the stability of Cdc6p is unaffected in APC mutants. We reasoned that expressing a stabilized allele of Cdc6p in a *cdc16* mutant might result in enhanced overreplication. While this particular effect has not been demonstrated, we are presently characterizing the effect of expressing the cdk 1-6 allele of Cdc6p in the *cdc16* background. We expect to find one of three things to be true: first, these cells may suffer from unregulated initiation; second, initiation itself may be impaired, which would in turn suggest a role for both the APC and the phosphorylation of Cdc6p in initiation; and third, cells may be arrested in G2 as a consequence of failing to degrade Cdc6p and other DNA replication proteins. Whatever the result, we expect that characterization of this phenotype will lead to a higher understanding of the control of DNA replication in *Saccharomyces cerevisiae.*

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Table 1. Oligonucleotides used for amplification and subcloning

Oligo Name	SEQUENCE
SE25	AATTGGCCGAGCTCC
SE26	AATTGGAGCTCGGCC
SE27	GATCCGGCATATGGGCGGCCTGCA
SE28	GGCCGCCCATATGCCG
SE37	GGCCAAAGCTTCAATTGTACCCCACCTC
SE45	GGATCCCATATGTCATTTGACGATGCTCCAG
SE46	CCAACTAAGCGTATCCCTTTGAAAAGAAAAAAGTTGC
SE47	CGATGCTCCAGCAGATGTTACACCAGAATC
SE48	CGACCTTTGAAAAGAAAAAAGCTGCAGTTTGGCTC
SE53	CTAGCGAACAAAAGTTGATTTCTGAAGAAGATTTGC
SE54	CTAGGCAAATCTTCTTCAGAAATCAACTTTTGTTCG
SE61	GCGAGCTCTGAACTACATAGCC
SE62	TGACTAGTGGGCCGCTCTAGAACT
SE63	GAAACCTTTCCTTCACCCCGGGGGTTCCACTAGC
SE64	GCTAGTGGAACCCCCGGGGTGAAGGAAAGGTTTC
SE72	CCTCCACGACCTTTGGCAGCAGCAGCGTTGCAGTTCACAGAT
SE74	TATGTCAGCTATACCAATAACTCTGCA
SE75	GAGTTATTGGTATAGCTGACA
SE84	GCTCCAGCAGCGCCTCCACGA
SE97	GCTATACCAATAGCCCAACTAAGCGT
SE98	ACACCAGAATCAGCGCC AGAAAAACTG
SE99	TTGTCGCTCTCCGCGCCAC GCTCGAAG
SE100	CGGTTTCTGCTTGCGC CAACAAGAGGA
SE101	ACGCCAACTACTGCG CCGGT AAAGAAA

SE102	GGCGGCCGCAATACGACTCACTATAGGGATGTCAGCTATACCAATA
SE103	TTGAAACCTTTCCTTCACTAGTCAGCTAATAGATTTATT
SE108	GGATCCTAATACGACTCACTATAGGAACAGACCACCATGGCAGCTATACCAATAACTCCAACT
SE128	GGGGATCCGTATGTCAGCTATACCAATAACTCC
SE129	GGGGATCCGTATGTCAGCTATACCAATAGCTC
SE130	GGGGATCCGTATGTCATTTGACGATGCTCC
SE131	GGGGATCCGTCTGCAGTTTGGCTCACAG
SE132	GGGGGATCCTCACTAGTGAAGGAAAGGTTTCAAAATTGAAAT
SE133	GGGGGTCGACTCACTAGTGAAGGAAAGGTTTCAAAATTGAAAT
SE134	GGATCCTAATACGACTCACTATAGGAACAGACCACCATGGCAGCTATACCAATAACTCTGCAG
SE135	GGATCCTAATACGACTCACTATAGGAACAGACCACCATGGCATTTGACGATGCTCCAGCAACG
SE136	GGATCCTAATACGACTCACTATAGGAACAGACCACCATGGCAGCTATACCAATAGCGCCAACT

Table 2. Oligonucleotides used for mutagenesis

Sites mutated	Mutation	Oligo for mutagenesis
ΔN terminus	Δ8-48	
Δ3-16	Δ3-16	SE45
Δ13-26	Δ13-26	SE46
Δ23-36	Δ23-36	SE47
Δ33-46	Δ33-46	SE48
NLS	K29A, R30A, K31A, K32A	SE72
cdk1	T7A	SE97
cdk2	T23A	SE84
cdk3	S43A	SE98
cdk4	T135A	SE99
cdk5	S354A	SE100
cdk6	S372A	SE101

Table 3. Plasmids for in vitro ubiquitination and stability studies

Sites mutated	Bluescript construct	GAL plasmid
wild type	pELS91	pELS147
Δ8-48	pELS144	pELS148
Δ3-16	pELS92	pELS149
Δ13-26	pELS93	pELS150
Δ23-36	pELS94	pELS151
Δ33-46	pELS95	pELS152
NLS	pELS105	pELS156
cdk1	pELS164	pELS176
cdk2	pELS193	pELS193
cdk3	pELS177	pELS177
cdk4	pELS165	pELS178
cdk5	pELS166	pELS179
cdk6	pELS167	pELS194
cdk1+2	pELS187	pELS198
cdk2+3	pELS162	pELS180
cdk1+3	pELS197	pELS199
cdk1, 2, 3	pELS188	pELS200
cdk 5+6	pELS163	pELS181
cdk1 to cdk6	pELS159X	pELS175

Table 4. Plasmids for two hybrid assays

	5' oligo	3' oligo	plasmid
wild type	SE128	SE133	pELS211
Δ8-48	SE131	SE133	pELS212
Δ3-16	SE130	SE133	pELS213
Δ13-26	SE129	SE132	pELS226
Δ23-36	SE128	SE133	pELS214
Δ33-46	SE128	SE133	pELS215
NLS	SE128	SE133	pELS224
cdk1+2	SE129	SE133	pELS217
cdk2+3	SE128	SE133	pELS216
cdk1+3	SE129	SE132	pELS225
cdk1, 2, 3	SE129	SE132	pELS229
cdk 5+6	SE128	SE132	pELS222
cdk1 to cdk6	SE129	SE132	pELS218

Figure 1. Interaction of mutant Cdc6p with Cdk1p. Strain BJ2168 was transformed with plasmids pELS71 (wildtype), pELS72 (Δ3-16), pELS73 (Δ13-26), pELS74 (Δ23-26), and pELS75 (Δ33-46). Cells were grown overnight in synthetic medium prepared with raffinose and lacking leucine. Expression of Cdc6 proteins was induced for 4 hours with 2% galactose. Extracts were prepared and immunprecipitations were carried out as described in Materials and Methods. Top panel: 10 μg of protein was electrophoresed on a 10% gel, blotted to PDVF membrane (Millipore), and probed with anti-myc monoclonal antibody 9E10. The apparent molecular weight of Cdc6-Myc9p is 120 kD. Bottom two panels: 9E10 immunoprecipitates were electrophoresed on a 12.5% and blotted to PDVF membrane. The top half of the membrane was probed with anti-myc monoclonal antibody 9E10 (middle panel), and the bottom half of the membrane (bottom panel) was probed with anti-Cdk1p polyclonal antibody RAA1. Protein blots were developed with ECL reagents (Amersham).

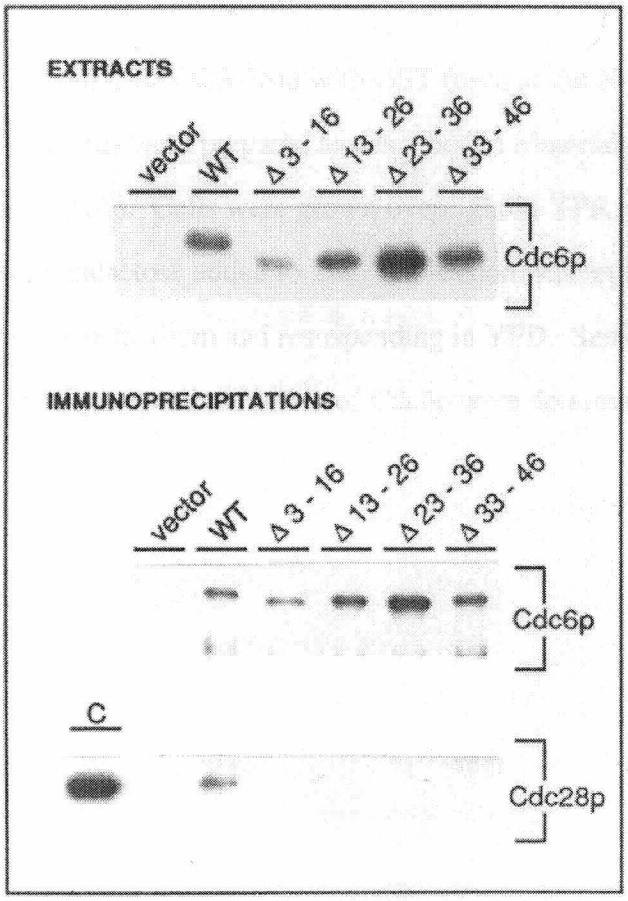

EXTRACTS
vector
WT
Δ 3 - 16
Δ 13 - 26
Δ 23 - 36
Δ 33 - 46
Cdc6p
IMMUNOPRECIPITATIONS
vector
WT
Δ 3 - 16
Δ 13 - 26
Δ 23 - 36
Δ 33 - 46
Cdc6p
C
Cdc28p

Figure 2. Stability of GST-Cdc6p and GST-(Δ23-36) Cdc6p. Plasmids bearing wild type Cdc6p and Cdc6(Δ23-36)p with GST fused at the N-terminus and 9xMyc fused at the C-terminus were prepared as described in Materials and Methods, and transformed into strain BJ2168. Cells were grown overnight in YPR, and expression was induced for 1 h with solid galactose added to 2%. Expression was repressed by removing cells from galactose-raffinose medium and resuspending in YPD. Samples were taken at the times indicated in the Figure, and the levels of Cdc6p were determined as in Figure 1.

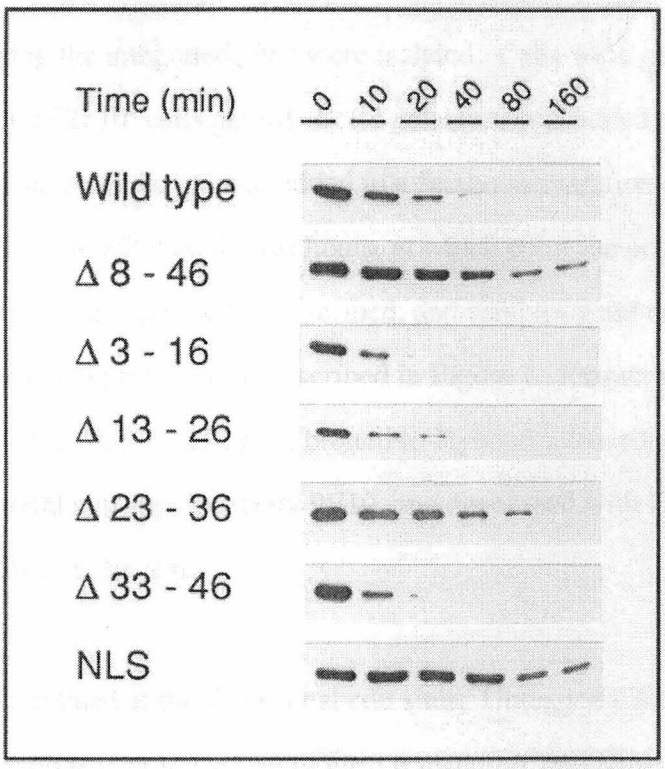
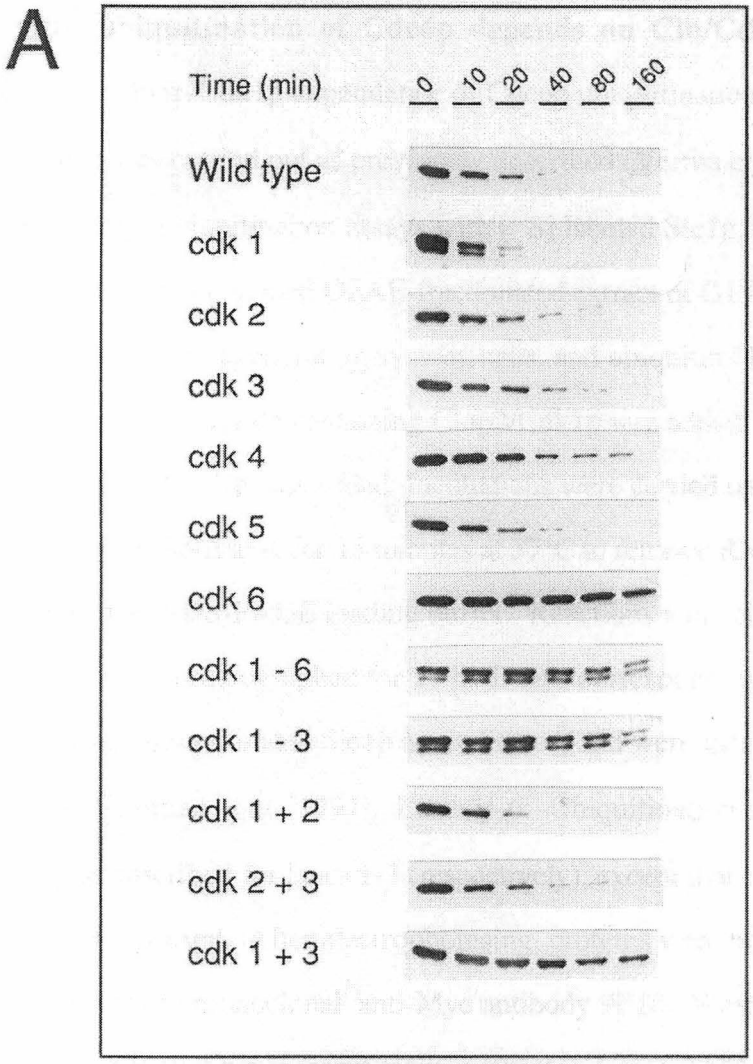

Time (min)
0
10
20
40
80
160
Wild type
Δ 8 - 46
Δ 3 - 16
Δ 13 - 26
Δ 23 - 36
Δ 33 - 46
NLS

Figure 3. Stability of Cdc6p point mutants.

(A) Strain BJ5459 was transformed with integrating plasmids bearing wild type or mutant *CDC6* tagged at the C-terminus with 9xMyc and driven by the *GAL* promoter. Transformants carrying the integrated gene were isolated. Cells were grown overnight in YPR to a cell density of $2x10^6$ cells per ml. Solid galactose was added to 2%, and cells were incubated for 2 h. Nocodazole was added to a final concentration of 15 μg/ml, and cells were incubated for an additional three hours, at which point the population was arrested. Transcription was repressed as described, and samples were taken at the times indicated in the Figure and processed as described in Figure 1. Extract was electrophoresed on 7.5% SDS-PAGE gels, blotted to Hybond nitrocellulose (Amersham), probed with monoclonal anti-myc antibody 9E10, and developed with ECL reagents. Mutations are described in the text.

(B) Cdc6p is phosphorylated at the C-terminal cdk sites. Untagged *CDC6* was amplified and [^{35}S]-labeled Cdc6p was produced by in vitro translation, and phosphorylation reactions were carried out as described in Materials and Methods. Protein was electrophoresed on a large 7.5% SDS gel (18 cm long), electrophoresed at 30 mA for 3 h, dried and autoradiographed.


A
Time (min)
0
10
20
40
80
160
Wild type
cdk 1
cdk 2
cdk 3
cdk 4
cdk 5
cdk 6
cdk 1 - 6
cdk 1 - 3
cdk 1 + 2
cdk 2 + 3
cdk 1 + 3

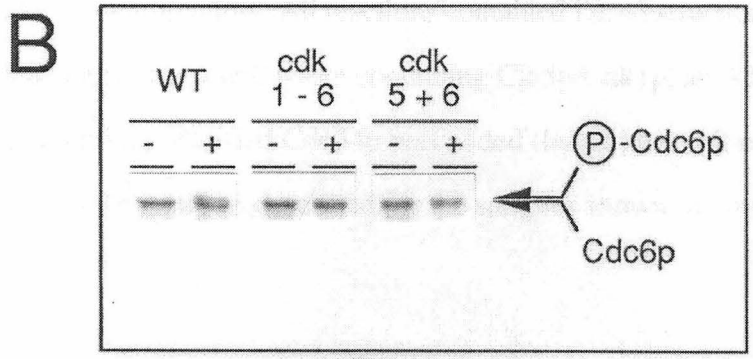

B
WT
cdk
1 - 6
cdk
5 + 6
-
+
-
+
-
+
P-Cdc6p
Cdc6p

Figure 4. In vitro ubiquitination of Cdc6p depends on Clb/Cdk1p and Cdc34p. Lanes 1-6: Clb5p/Cdk1p dependence of Cdc6p ubiquitination. Lanes 1-3: Ubiquitination of Sic1p was carried out as previously described (Verma *et al.*, 1997a). Lanes 1 through 3: in vitro ubiquitination assays with [^{35}S] labeled Sic1p, produced by in vitro translation. All reactions contained DEAE-fractionated extract of G1 arrested RJD885 cells, purified Cdc34p, an ATP regenerating system, salts, and ubiquitin. Lane 1, no kinase added; lane 2, insect cell lysate containing Clnp2/Cdk1p was added; lane 3, insect cell lysate containing Clb5p/Cdk1p was added. Incubations were carried out for 1 h at 25°C, treated with 20 μg/ml RNAse A for 15 minutes at 37°C to remove RNA-protein adducts, and quenched with SDS-PAGE loading buffer. Reactions were electrophoresed, and the gel was dried and autoradiographed for 17 h. Bands corresponding to Sic1p, phosphorylated Sic1p, and ubiquitinated Sic1p are indicated, and were identified based on previous descriptions (Feldman *et al.*, 1997). Lanes 4-6: Ubiquitination of Cdc6p. Reactions are exactly as described for lanes 1-3 (respectively), except that cold, in vitro translated Cdc6-Myc9p was used. After electrophoresing, proteins were blotted to Hybond nitrocellulose and probed with monoclonal anti-Myc antibody 9E10. Westerns were developed with the Super Signal luminescent reagent (Pierce). Lanes 7 and 8: Cdc34p dependence of Cdc6p ubiquitination. All reactions contained DEAE-fractionated extract of G1 arrested RJD885 cells, insect cell lysate containing Clb5p/Cdk1p, an ATP regenerating system, salts, and ubiquitin. Purified Cdc34p was added (lane 8) or omitted (lane 7). Samples were processed exactly as described for the samples shown in lanes 4 - 6.

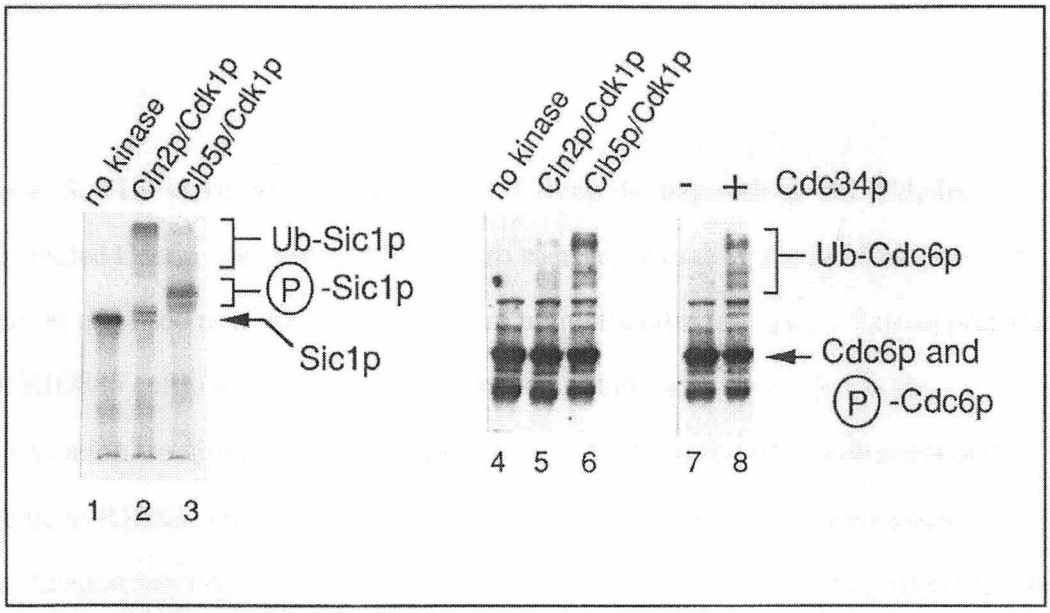

no kinase
Cln2p/Cdk1p
Clb5p/Cdk1p
Ub-Sic1p
P -Sic1p
Sic1p
1 2 3
no kinase
Cln2p/Cdk1p
Clb5p/Cdk1p
- + Cdc34p
Ub-Cdc6p
Cdc6p and
P -Cdc6p
4 5 6
7 8

Figure 5. In vitro ubiquitination of Cdc6p is dependent on Cdc4p. [^{35}S] labeled Cdc6p was prepared by in vitro translation as described in Materials and Methods, and used as a substrate for in vitro ubiquitination reactions. Extract prepared from RJD885 cells (wild type for ubiquitination components) was used in the ubiquitination reactions in lanes 2 through 6. Extract from RJD893 cells (*cdc4* and isogenic to RJD885) was used in reactions in lanes 7 through 12. The complete ubiquitination reaction (lanes 4 and 8) included DEAE-fractionated extract of G1 arrested cells, purified Cdc34p, insect cell lysate containing Clb5p/Cdk1p kinase, an ATP regenerating system, salts, and ubiquitin. The reactions in lanes 6, 11, and 12 were supplemented with recombinant Cdc4p purified from insect cells. Cdc34p and Clb5p/Cdk1p were omitted where indicated.

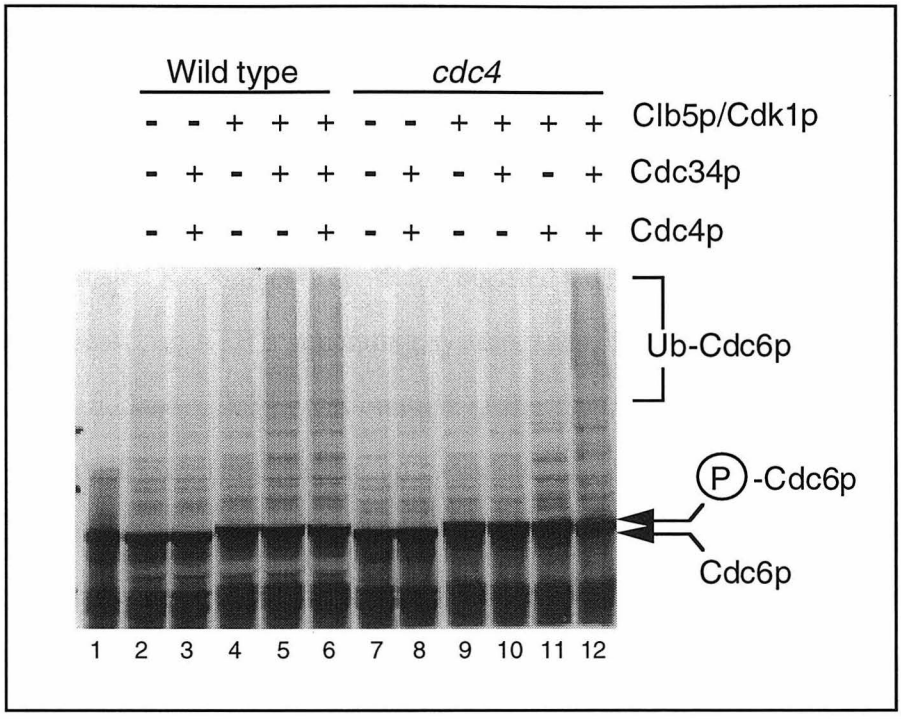

Wild type
cdc4
- - + + + - - + + + +
Clb5p/Cdk1p
- + - + + - + - + - +
Cdc34p
- + - - + - + - - + +
Cdc4p
Ub-Cdc6p
P -Cdc6p
Cdc6p
1 2 3 4 5 6 7 8 9 10 11 12

Figure 6. In vitro ubiquitination of Cdc6p mutants. Wild type and mutant Cdc6-Myc9p were produced by in vitro translation as described in Materials and Methods, and ubiquitination reactions were carried out as described. For each pair of samples, the reaction in the right-hand lane was a complete ubiquitination reaction (DEAE-fractionated extract of G1 arrested RJD885 cells, partially purified Cdc34p, insect cell lysate containing Clb5p/Cdk1p kinase, an ATP regenerating system, salts, and ubiquitin). For the reactions run in the left-hand lane, Cdc34p was omitted. After the ubiquitination reaction, samples were treated with RNAse (as in Figure 4), mixed with SDS-PAGE loading buffer and electrophoresed on 7.5% gels. Proteins were blotted to Hybond nitrocellulose, probed with 9E10 antibody, and the Westerns were developed with the Super Signal luminescent reagent (Pierce).

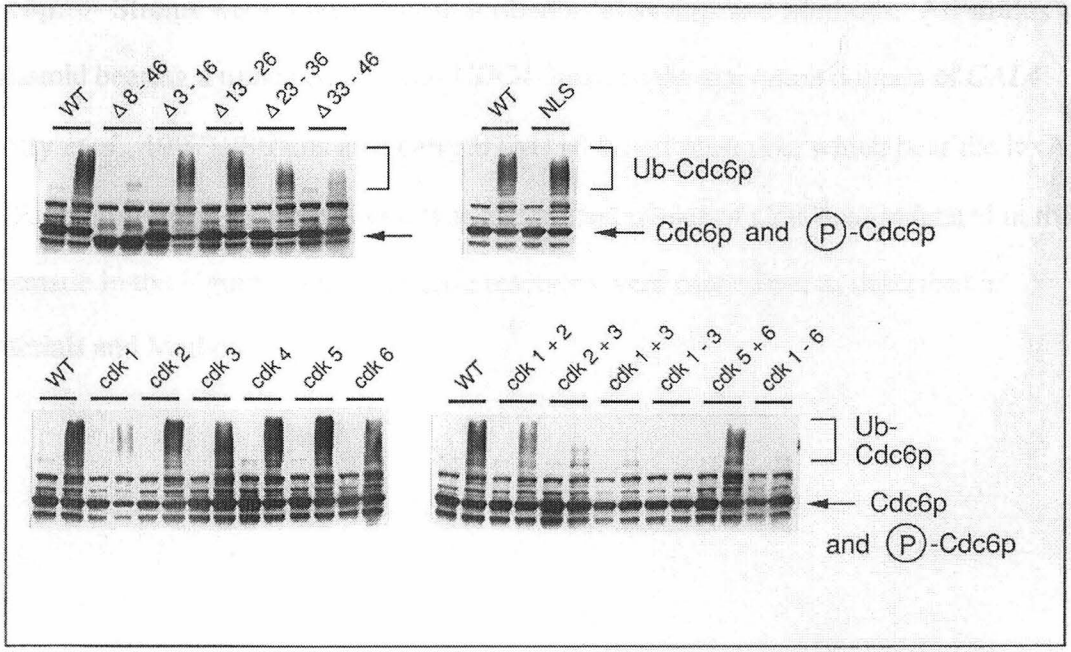

WT
Δ 8 - 46
Δ 3 - 16
Δ 13 - 26
Δ 23 - 36
Δ 33 - 46
WT
NLS
Ub-Cdc6p
Cdc6p and (P)-Cdc6p
WT
cdk 1
cdk 2
cdk 3
cdk 4
cdk 5
cdk 6
WT
cdk 1 + 2
cdk 2 + 3
cdk 1 + 3
cdk 1 - 3
cdk 5 + 6
cdk 1 - 6
Ub-
Cdc6p
Cdc6p
and (P)-Cdc6p

Figure 7. Two hybrid interaction of Cdc4p with wild type and mutant Cdc6p. Strains were prepared as described in Materials and Methods. All strains carry a plasmid bearing a truncated allele of *CDC4* fused to the activation domain of *GAL4* (Drury *et al.*, 1997). Strains also carry BTM116-based plasmids, which bear the lexA DNA binding domain fused to wild type and mutant alleles of *CDC6*, as indicated in the schematic in the Figure. β-galactosidase reactions were carried out as described in Materials and Methods.

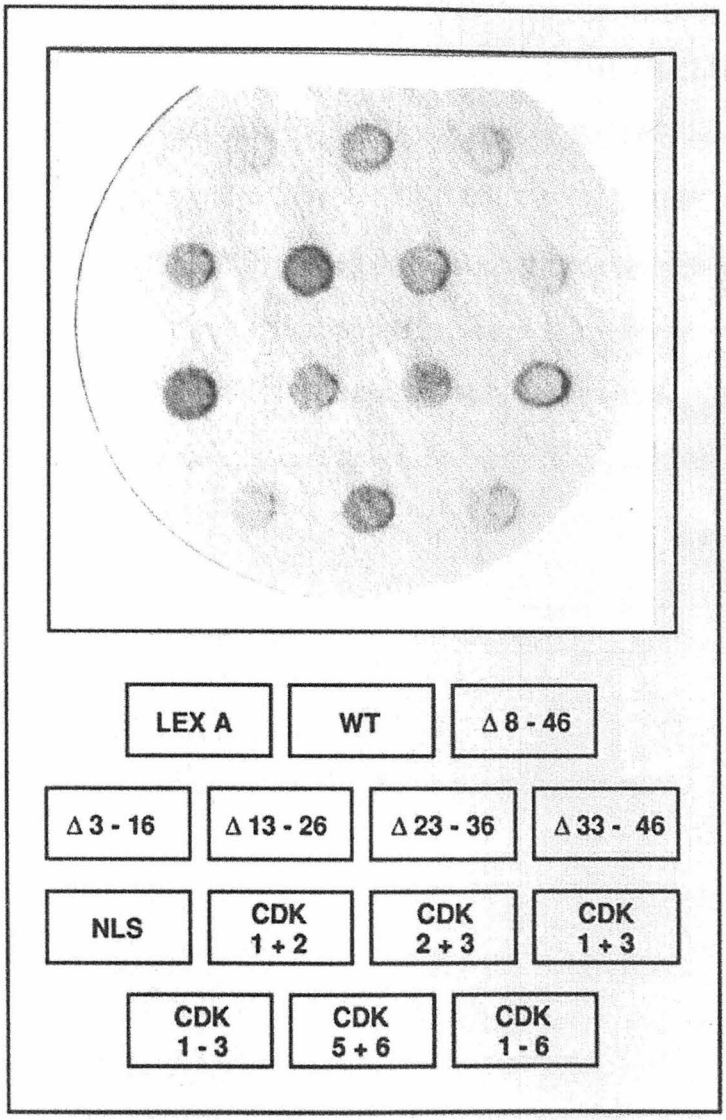

LEX A
WT
Δ 8 - 46
Δ 3 - 16
Δ 13 - 26
Δ 23 - 36
Δ 33 - 46
NLS
CDK 1 + 2
CDK 2 + 3
CDK 1 + 3
CDK 1 - 3
CDK 5 + 6
CDK 1 - 6

Figure 8. Hyperinstability of Walker A motif mutant (K114E) Cdc6p. Stability assays were carried out as in Figure 3, using strain BJ2168 transformed with YGpKTU6, which bears a copy of wild type *CDC6* fused at the N-terminus to *GST,* and YGpME6, differing from YGpKTU6 only in that lysine 114 has been mutated to glutamate (K114E). Samples were taken at the time points indicated in the Figure. 10μl of protein was electrophoresed on 7.5% SDS-PAGE gels, blotted to nitrocellulose, and probed with polyclonal GST antibody. Westerns were developed with ECL reagents.

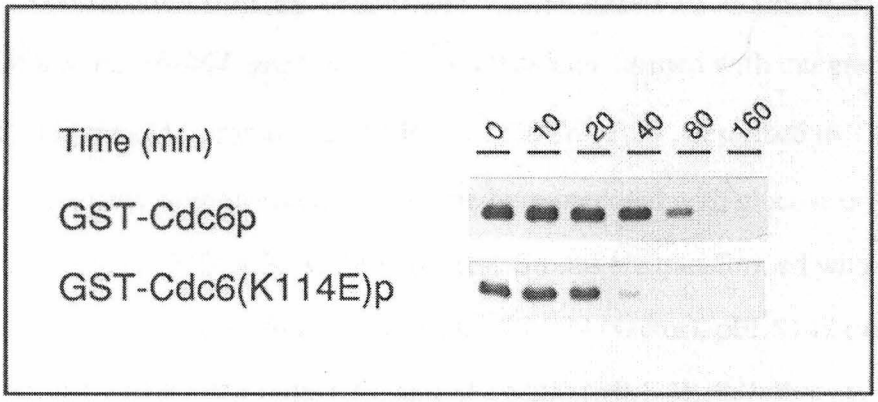
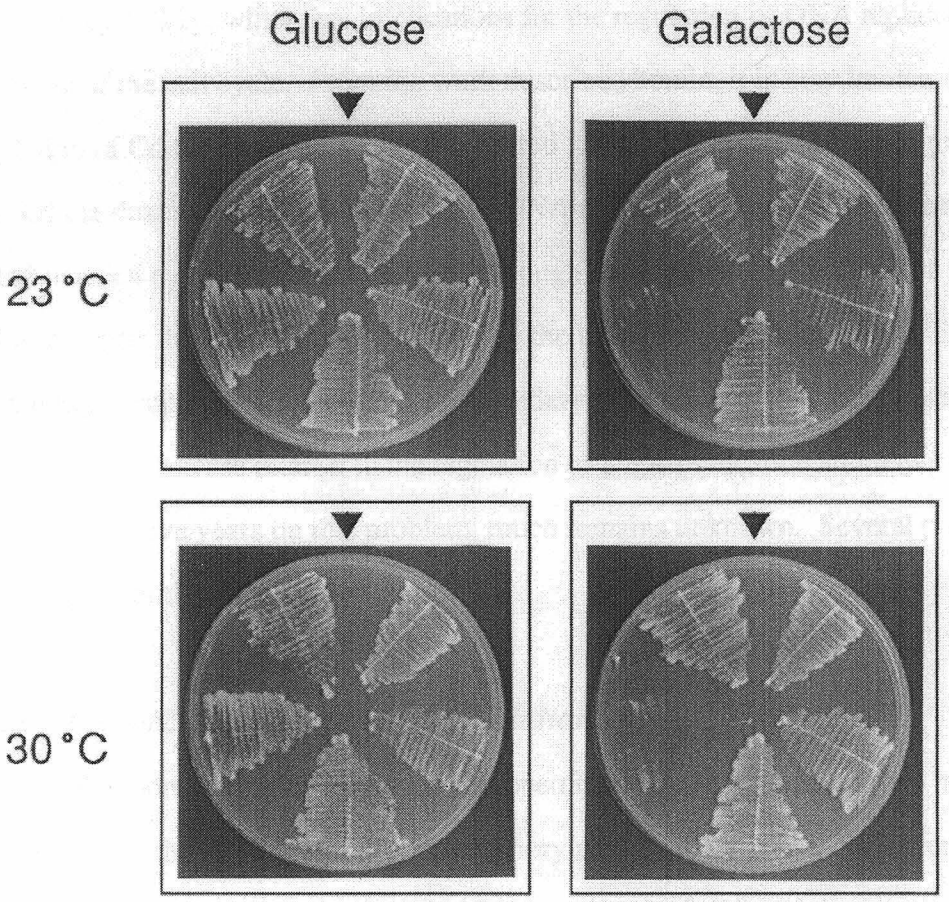

Time (min)
0
10
20
40
80
160
GST-Cdc6p
GST-Cdc6(K114E)p

Figure 9. Overproduction of Cdc6(cdk1-6)p is lethal in a *cdc16* strain. **KHY303** (*Mat a cdc16-264 ura3 trp1 Gal+*) was transformed with integrating plasmids bearing the *GAL* promoter and alleles of *CDC6-Myc9* , described in Table 3. Purified transformants were streaked on ura - medium prepared with glucose or galactose, and incubated at either 23°C or 30°C for two days. Strains are transformed with the following plasmids (clockwise from the arrow): pELS89 (vector), pELS147 (wildtype), pELS148 (Δ8-48), pELS159X (cdk 1-6), and pELS200 (cdk1-3).


Glucose
Galactose
23 °C
30 °C

Chapter 4: Future Directions

At the outset of studying Cdc6p, I wished to understand the role of this protein in DNA replication. During the course of the work, I found that Cdc6p interacts with and is phosphorylated by Cdk1p, which has implications for the regulation of DNA replication over the course of the cell cycle. From the work described herein, it is now known that the phosphorylation of Cdc6p by the Clb/cdks is required for the cell cycle specific degradation of Cdc6p. Of the dozens of proteins involved in DNA replication, few have been shown to be regulated in a cell cycle dependent fashion, although many have been studied for such an effect. This suggests that degradation of Cdc6p by the ubiquitination machinery is likely to be one of the key components of the block to secondary replication in the later phases of the cell cycle. In spite of intense interest in the regulation of DNA replication and remarkable progress in the past five years on this problem, much remains unknown. Several possible lines of inquiry are outlined below.

Substrates of Clb/Cdk1p Targeted in Initiaiton

One of the more intriguing questions still open in the DNA replication field is the following: What are the substrates that are phosphorylated by the S phase cyclin dependent kinases in an event that causes the initiation of DNA replication? When I discovered that Cdc6p associated tightly with Cdk1p, it seemed likely that phosphorylation of Cdc6p by Clb/Cdk1p would be involved in the initiation of DNA replication. While such requirements for initiation have not been proven, they have also not been ruled out. One model for the role of the Clb5,6p/Cdk1p and Dbf4p/Cdc7p kinases in the initiation of DNA replication is that multiple components of the prereplicative complex are phosphorylated, and the prereplicative complex is thus destabilized in a step required for origin firing. In

this scenario, there are no single phosphorylations that are crucial for origin firing, nor is there just one target of the kinases. This circumstance would explain why no one has managed to identify the crucial targets, since the extensive mutation of several genes and analysis of these mutant alleles in vivo would be required.

In order to address this question, I propose that all of the canonical cdk sites in all of the relevant proteins be mutated, cloned under a conditional promoter, and expressed in various combinations in vivo. One caveat to this approach is the observation that the substrates of the cyclin dependent kinases are not always phosphorylated at canonical cdk sites. However, mutation of the canonical sites of Sic1p and Cdc6p has generated mutant proteins that behave considerably differently in vivo than the wild type protein. A second caveat is that such an analysis would be exhaustive beyond the point of tractability, but an analysis of the potential cdk sites in the ORC subunits, the Mcm family members, Cdc45p, Clb5p, Clb6p and Dbf4p suggests that this is not the case (see Table 1). Strikingly, several of the licensing proteins, Orc2p, Orc6p, Cdc6p, Mcm3p, and Dbf4p, have a number of potential cdk phosphorylation sites, and the majority of the remaining licensing proteins have no canonical sites at all. Furthermore, the sites present in the candidate substrates are clustered: Orc2p is a 620 residue protein, but the phosphorylation sites are clustered in two patches of 55 and 25 amino acids in length. Orc6p, with 443 residues, possesses four canonical cdk sites within a 40 residue patch. The clusters of phosphorylation sites in Cdc6p have been discussed extensively in Chapter 3, and mutation at each of these patches gives an in vivo effect. Mcm3p, a 961 residue protein, shows five potential phosphorylation sites within an 84 residue patch. Dbf4p, observed in our lab to be an unstable protein, has two potential phosphorylation sites right at the N-terminus. For all of these proteins, there are many more prolines than potential cdk sites, suggesting that the clustering of the cdk sites does not derive from a constraint of protein structure. I propose that the clusters are not a remarkable coincidence but occur due to a requirement imposed

by evolution. A third caveat of this approach is that conditional inactivation of wild type alleles might be required to see an effect. This would be simple enough if only one protein were involved, but multiple proteins are proposed to be involved, and preparing strains with multiple mutations is somewhat limited by synthetic lethality effects, both previously observed and possible. Inactivating the transcription of initiation proteins by using strains defective in the factors required for SCB and MCB transcription might be one approach for inactivating wildtype initiation proteins. My belief is that the right combination of phosphorylation deficient alleles would be sufficiently deleterious in the presence of wildtype proteins to cause a detectable effect, and that there are enough methods to surmount the wildtype background problem so as to generate a convincing result.

The Mechanism of Initiation

While a number of the components required for initiation have been identified, much remains unknown about the mechanism of initiation. Arguably this would be best served by the use of an in vitro system, but this has been long sought and only recently reported (Pasero, P., Braguglia, D., and Gasser, S.M. (1997) *Genes Dev.* **11**, 1504-1518). DNA replication in this in vitro system is ORC-dependent, origin-dependent, and replication appears to be semiconservative. However, this system also depends on intact yeast nuclei, which means that immunodepletion experiments are not possible. While the existence of this in vitro system is a promising beginning, the advantages offered over in vivo observations are somewhat limited. An in vitro DNA replication system that faithfully mimics in vivo events and is comprised of purified components is the ideal goal. Such an in vitro system was developed for SV40 (simian virus 40) DNA replication, and this allowed great insights about the assembly of DNA polymerases at the replication fork, and the mechanism that allows for the switching between DNA polymerase α, which is associated with primase and is the only polymerase which will synthesize nascent DNA,

and DNA polymerase δ, thought at the time to carry out the bulk of DNA synthesis in vivo. Producing an in vitro system with purified proteins that is representative of in vivo yeast DNA replication is likely to be a formidable task, especially as initiation in yeast is highly regulated, and a true in vitro system would not only require the purification of a variety of factors but the preparation of these factors in the appropriate phosphorylation states. In addition, all of the factors involved may not be known, which in turn makes fractionation a necessary component of an approach to gaining an in vitro system.

The first great result of establishing a minimal in vitro system would be just that: demonstrating exactly which proteins are required for origin-dependent, ORC dependent, kinase-dependent, semi-conservative DNA replication. The availability of a minimal in vitro system would then be a great advantage in studying the steps in initiation at yeast origins. In spite of dozens of new papers describing the involvement of Cdc6p and friends in initiation, a clear biochemical function for Cdc6p and many other factors is undescribed. Some of the mechanistic questions that could be answered by an in vitro system include: What proteins are required for the formation of an open complex? What cellular proteins are directly involved in loading the DNA polymerases at the forks of the open complex? How does the Mcm helicase operate, and why are six subunits required? Is the removal of Cdc6p from the prereplicative complex a crucial step in initiation, as it appears to be by some methods of analysis? The answers to these and other questions lay at the end of this road less traveled.

Once and Only Once Replication

One of the more controversial findings to be reported in the DNA replication field in the past few years is that anaphase promoting complex mutants undergo reinitiation of DNA. While secondary origin firing in G2 was shown to require the initiation proteins Cdc6p and Mcm5p, and to be both origin-directed and semiconservative, this finding is

especially provocative because DNA replication occurs in the presence of high Clb/Cdk1p kinase activity, which in turn has been shown by several approaches to normally block spurious initiation. It is known that in G2 cells, Cdc6p and Dbf4p are degraded and that the Mcms are exported from the nucleus, presumably due to high Clb/cdk kinase activity. Not only this, but the transcription of DNA replication genes that normally occurs in G1 is absent. Inhibiting Clb/cdk activity in G2, which allows for an unscheduled round of DNA synthesis, is thought to reverse all of the effects of high Clb/cdk activity, and has been explicitly shown to allow the synthesis of G1 transcripts. In light of this, the finding that APC mutants rereplicate their DNA is even more surprising. My expectation in this regard is that prereplicative complexes are not lost at origins in the APC mutants, although in vivo footprinting studies performed outside the context of the rereplication work suggest that this is not the case. My opinion here stems from the observation that in *cdc6* mutants which overreplicate their DNA, the Mcms are still found associated with origins although the bulk of the Mcms are exported at the G1/S transition. Origins may remain licensed rather than become relicensed, and this proposition is testable with the techniques discussed in Chapter 1. If the anaphase promoting complex is required for revoking the license to replicate, it should be possible to find suppressors of this phenotype, possibly by evaluating defective alleles of known initiation proteins. In addition, it should also be possible to find alleles of initiation genes that when transcribed exacerbate the rereplication phenotype. Although I have yet to prove it, I may have already accomplished this by generating an allele of Cdc6p which is toxic in an APC mutant.

Epilogue

Of the questions outlined here, my personal preference would be to investigate the targets of the S phase cyclin dependent kinases, both in the initiation of DNA replication and suppressing secondary initiation within a given cell cycle. It is perhaps a testament to my tenacity or maybe just single-mindedness that leads me believe that I would enjoy developing the research presented in this thesis for my entire career. In the interest of breadth, however, I will pursue other things and leave these lines of inquiry to other, unsuspecting graduate students. It remains possible that in my future work on the proteasome, the research will lead me back to DNA replication. As written by Robert Hunter in the song Scarlet Begonias, "Once in a while you get shown the light in the strangest places if you look at it right." This song was made famous by the Grateful Dead, and is here quoted by the Grateful Finished.

Table 1. Cdk sites in licensing proteins

Protein	Canonical cdk sites	Clb/Cdk1p phosphoryl-ation	Dbf4p/Cdc7p phosphoryl-ation
Orc1p	ARHTN S_{304}PRK RGRKI	N.D.	N.D.
Orc2p	NDILS S_{16}PAK SRNVT KSRNV T_{24}PKR VDPHG ALKPK T_{70}PSK APRKR PPEPA T_{174}PSK KSLT NHDFT S_{188}PLK QIIMN YKDST S_{206}PGK LTLSR	N.D.	N.D.
Orc3p	none	N.D.	N.D.
Orc4p	none	N.D.	N.D.
Orc5p	none	N.D.	N.D.
Orc6p	SLSNS S_{104}PMK QFAWT FAWTP S_{114}PKK NKRSP KKNKR S_{121}PVK NGGRF NQLFG T_{144}PTK VRKSQ	***	N.D.
Cdc6p	SAIPI T_{7}PTK RIRRN DDAPA T_{23}PPR PLKRK VTPES S_{43}PEK LQFGS KRFLL S_{354}PTR GSLNS LTPTT S_{372}PVK KSYPE	*****	N.D.
Cdc45p	none	N.D.	N.D.
Mcm2p	none	N.D.	*****
Mcm3p	ALSKR S_{761}PQK SPKKR RSPQK S_{765}PKK RQRVR ASNSG S_{781}PIK STPRR SPIKS T_{786}PRR STASS LGLRV S_{845}PRR REHLH	N.D.	***
Mcm4p	SQQSS S_{7}PTK EDNNS TSDLS S_{145}PRR IVDFD	***	*
Mcm5p	none	N.D.	-
Mcm6p	none	N.D.	*****
Mcm7p	none	N.D.	-
Dbf4p	MV S_{3}PTK MIIRS KMIIR S_{11}PLK ETDTN	N.D.	*****
Clb5p	none	N.D.	N.D.
Clb6p	none	N.D.	N.D.

Appendix: Role of Multifunctional Autonomously Replicating Sequence Binding Factor 1 in the Initiation of DNA Replication and Transcriptional Control in *Saccharomyces cerevisiae*

(Peter R. Rhode, Suzanne Elsasser, and Judith L. Campbell, *Molecular and Cellular Biology* 12, 1064-1077 (1992))

Role of Multifunctional Autonomously Replicating Sequence Binding Factor 1 in the Initiation of DNA Replication and Transcriptional Control in *Saccharomyces cerevisiae*

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Autonomously replicating sequence (ARS) binding factor 1 (ABF1) is an abundant DNA-binding protein that specifically recognizes the motif RTCRYN$_5$ACG at many sites in the yeast genome, including promoter elements, mating-type silencers, and ARSs. Mutational analysis of these sites suggests that ABF1 is involved in constitutive and carbon source-regulated transcriptional activation, transcriptional silencing, and ARS activity. To better assess the role of ABF1 in DNA replication and transcriptional control, temperature-sensitive lethal mutations in the *ABF1* gene were isolated. Several of the *abf1*(Ts) strains show rapid growth arrest at the nonpermissive temperature. At the semipermissive temperature, these strains show an ARS-specific defect in the mitotic stability of *ARS-CEN* plasmids, such that the *abf1* mutants show defects in ARS function identical to those of mutants bearing the mutations in the *cis*-acting ABF1 binding sites analyzed previously by numerous investigators. Flow cytometric analysis and in vivo DNA labeling experiments on an α-factor synchronized *abf1*(Ts) strain showed that at the nonpermissive temperature, these cells fail to progress efficiently from G_1 through S phase and synthesize DNA at 25% of the level seen in the isogenic *ABF1* strain. RNA synthesis is also reduced in the *abf1*(Ts) strains. In addition, transcriptional activation by an ABF1 binding site upstream activation sequence is completely defective in an *abf1*(Ts) strain at the semipermissive temperature. These phenotypes provide evidence that the same protein, ABF1, functions in the initiation of DNA replication and transcriptional activation.

The initiation of DNA replication requires the concerted action of many proteins at discrete replication origins along each chromosome. In prokaryotic and viral replicons, a sequence-specific DNA-binding protein called the initiator interacts with and unwinds the origin region prior to the initiation of bidirectional replication by the DNA polymerase-primase complex. In many cases, auxiliary DNA-binding proteins are also required for efficient origin activity (see reference 23 for a review). Current efforts have focused on characterizing similar initiation factors and their mechanisms of action in eukaryotic chromosomal replication, whereby multiple origins initiate in a specific temporal pattern during the S phase of the cell cycle.

In the yeast *Saccharomyces cerevisiae*, origins of DNA replication were initially characterized as autonomously replicating sequences (ARSs) by their ability to act in *cis* to allow extrachromosomal maintenance of plasmid DNA. Direct evidence that a subset of ARSs (though not all) function as chromosomal origins has come from two-dimensional gel analysis of replication intermediates (5, 40, 41). Sequence requirements for origin function in plasmids and in the chromosome reveal that yeast ARSs are complex (see reference 14 for a review). Comparison of more than 20 ARSs identified two common features: high A+T content and a common consensus sequence (WTTTATRTTTW, where W is A or T and R is A or G), referred to as domain A (7). Mutational analysis of domain A has shown that it is essential but not sufficient in most contexts for plasmid ARS function (15, 45). In addition, sequences (referred to as domain B) 3′ to the T-rich strand of the core consensus contain multiple functionally redundant elements that are required for ARS activity. The nature of these elements varies between different ARSs and may include close matches to the core consensus (56), specific protein recognition sites (8, 24, 29, 62, 68), and sequences that allow DNA bending (69, 75) or melting (71). By analogy to prokaryotic and viral systems, these findings suggest that initiator and auxiliary proteins interact with the core consensus and domain B elements, respectively, to facilitate DNA unwinding prior to the initiation of DNA replication.

A number of proteins that bind to ARS elements have been characterized (8, 24, 29, 39, 60, 62, 68, 72). Recently, single-stranded-DNA-binding proteins, ACBP (39) and ssARS-T (60), have been identified on the basis of specific interaction with the T-rich strand of the ARS core consensus. Although these proteins do not appear to be functionally equivalent to replication initiators such as simian virus 40 T antigen, it has been speculated that they may participate in an unwound origin-protein complex. Several other DNA-binding proteins with undefined sequence specificity have been shown to interact with regions immediately flanking the core consensus sequence also required for optimal ARS activity (24, 72). One of these proteins, DBF-A, copurifies with an ATP-dependent helicase activity, further suggesting a role in DNA replication (72).

Several laboratories have independently identified an abundant DNA-binding protein that interacts with the sequence motif RTCRYN$_5$ACG in either the 3′ or 5′ flanking region of domain A in a subset of ARS elements (8, 24, 29, 30, 31, 62, 68). Site-directed mutagenesis of the ABF1 binding site at *ARS121* resulted in a large decrease in the stability of plasmids bearing this ARS (74), while deletions of this site at *ARS1* (24, 67, 69) and the *HMRE* ARS (4) had a smaller but detectable effect on *ARS* plasmid stability. In

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addition, Walker et al. (73) showed that the ABF1 binding site enhances the activity of *ARS121* in an orientation-independent manner and at distances up to 1.2 kb from the ARS core consensus. These findings suggest that ABF1 plays an auxiliary role in the initiation of DNA replication similar to that of the mammalian transcription and viral replication proteins NFI/CTF (44) and NFIII/OCT-1 (55). In addition to their locations at ARSs, ABF1 binding sites are found at regions involved in transcription control, including the silencer elements of the nontranscribed mating-type loci *HMR* and *HML* (8, 24, 62, 68) and the promoters of as many as 80 genes (9, 21, 22, 27, 35, 36, 59). At *HMRE*, the ABF1 binding site in combination with either an ARS consensus sequence or the binding site for the transcription repressor/activator protein RAP1 is required for the transcriptional repression of the adjacent *HMR* locus (4, 47). In the promoters for a variety of genes, the ABF1 binding site acts as an upstream activating sequence (UAS) involved in constitutive or carbon source-regulated transcriptional activation, depending on its context (6, 9, 16, 21, 22, 27, 28, 34–36, 38, 64). In fact, the ABF1 binding site from the *HMRE* silencer can act as a UAS element in the context of a heterologous promoter (4, 9). As many as a dozen laboratories have independently identified DNA-binding proteins (TAF, GF1, SUF, and BAP1) that interact with these transcriptional elements (6, 16, 21, 22, 27, 35, 36, 38). Most of these proteins have been shown to be immunologically or genetically identical to ABF1 (6, 16, 28, 34, 64). However, the possibility also exists that a DNA-binding protein different from ABF1 interacts at these sites (2).

The fact that the *ABF1* gene is essential for cell viability (25, 34, 59) and that overproduction of ABF1 (58) results in arrested growth verifies the importance of this protein in yeast growth. To better understand the roles of ABF1 in DNA replication and transcription and to verify that a single protein is responsible for the diverse effects of the *cis*-acting sites, we have isolated conditionally lethal mutations in the *ABF1* gene. The requirements for ABF1 in ARS activity, cell cycle progression, and transcriptional activation were examined in strains containing these mutations. The results of these experiments provide evidence that the same protein, ABF1, plays a fundamental role both in the initiation of DNA replication at ARSs and in transcriptional control in yeast cells.

MATERIALS AND METHODS

Plasmids, strains, and growth media. Plasmid stability assays were performed on the *ARS1-CEN4* plasmid YCpG1 (67), the *ARS121-CEN3* plasmid YCp5AB121 (74), and the *H4 ARS-CEN3* plasmid YCp5H4, generated by replacing the *Eco*RI-*Hin*dIII *ARS121* fragment of YCp5AB121 with the *Eco*RI-*Hin*dIII *H4 ARS* fragments of pAB9 (3). An *ARS1-CEN3* plasmid, YCp5A1, was also constructed by replacing the *Eco*RI-*Hin*dIII *ARS121* fragment of YCp5AB121 with the *Eco*RI-*Hin*dIII *ARS1* fragment of YRp17. This plasmid gave the same loss rates as did YCpG1. The *CYC1-lacZ* reporter gene plasmid pLGΔB and the *4XARE-CYC1-lacZ* construct were described by Harshman et al. (37). The *ABS-CYC1-lacZ* construct was made by inserting the double-stranded oligonucleotide

GATCCATTTCGTCAAAAATGCTAAGAAATCTGCA
GTAAAGCAGTTTTTACGATTCTTTAG

containing the ABF1 binding site from *ARS1* into *Bam*HI-*Pst*I-digested pBluescript SK$^-$ (Stratagene) and then subcloning the *Bam*HI-*Sal*I fragment into pLGΔB linearized with *Bgl*II and *Sal*I. Plasmid YEp24-4c was isolated from a yeast genomic library in YEp24 as described previously (59) and contains a 9-kb insert that includes the *ABF1* gene. Plasmid pR1190 was constructed by replacing the 3.4-kb *Bam*HI-*Nde*I fragment containing the *URA3* gene in YCp19 with the 5.3-kb *Bam*HI-*Nde*I fragment containing the *ABF1* gene from YEp24-4c. For the structure-function studies shown in Fig. 8, a series of 3′ deletions in the *ABF1* gene, H2.6 to H0.7, was generated by restriction digestions and exonuclease III digestions. H2.6 contains the entire coding region of ABF1. H1.1 and H2.1 extend to the first and second *Bal*I sites in the coding region, respectively (nucleotides 1118 and 2090) (59). H1.6 extends to nucleotide 1613, and H0.7 extends to nucleotide 695. Transcriptional termination signals, included in a 1-kb fragment originating 40 bases upstream of the first translation termination codon, were subcloned 3′ to each of these deletions. Stop codons appear in every reading frame within the first 130 bases of the termination signals. These constructs, which carry the *ABF1* promoter (*Hin*dIII to the initiation codon), were subcloned into the shuttle vector pRS316 (*CEN6, H4 ARS, URA3*) (63).

Escherichia coli TG1 cells were used for routine cloning procedures. The yeast strains used in this study are described in Table 1. Strain JCA11 was constructed by transforming the JCA10 diploid with YEp24-4c, sporulating, and selecting for URA$^+$ LEU$^+$ haploid segregants. This strain was not viable on 5-fluoroorotic acid media as a result of the loss of the plasmid-borne *ABF1* gene. The *abf1*(Ts) alleles (*abf1-1*, *abf1-2*, and *abf1-5*) were integrated into the chromosome as follows: the 1.8-kb *Bam*HI fragment containing the *HIS3* gene was subcloned into the *Bgl*II site 5′ to the *abf1*(Ts) genes (or *ABF1* gene). Previously we had shown that this region was not essential for cell viability. DNA fragments carrying the *abf1*(Ts) or *ABF1* genes linked to *HIS3* were used to transform YNN281 and YNN282 to HIS$^+$ phototrophy. Replacement of the chromosomal *ABF1* gene was verified by Southern analysis. Transformants were also tested for complementation of temperature-sensitive growth by the YEp24-4c (*ABF1*$^+$) plasmid. Other strategies for integrating the mutant alleles proved unsuccessful. JCA55 was a haploid segregant from a cross between AJ116 *bar1-1* and JCA45 *abf1-1* that showed both temperature-sensitive growth (complemented by YEp24-4c) and sensitivity (shmoo formation and growth arrest) to 0.3 μg of α-factor per ml. Strain JCA50 was constructed by replacing the *abf1-5* allele of JCA55 with the wild-type *ABF1* gene by one-step gene replacement.

Strains carrying the temperature-sensitive lethal mutations were grown at 23 to 25°C as the permissive temperature, 30°C as the semipermissive temperature, and 37 to 38°C as the nonpermissive temperature. All media were prepared as described previously (59). Yeast DNA transformations were carried out by the lithium acetate method of Ito et al. (42).

Generation of *abf1*(Ts) alleles. The plasmid shuffling strategy described by Budd and Campbell (11) was used to isolate *abf1*(Ts) alleles. Briefly, the *ABF1* gene on the *TRP1-ARS1-CEN4* plasmid pR1190 was randomly mutagenized in vitro by incubation with hydroxylamine for either 3 or 4 h according to the procedure of Busby et al. (13). The plasmids treated for 3 h transformed *E. coli* cells at 5% the efficiency of unmutagenized pR1190, while the transformation efficiency of the 4-h sample was 0.7%. Samples of both mutagenized populations were used to transform JCA11, a yeast

TABLE 1. Strains

Strain	Genotype	Reference or source
JCA10	*MATa/α leu2-3,112/leu2-3,112 ura3-51/ura3-52 his3Δ200/his3Δ200 trp1Δ901/trp1Δ901 lys2-801/LYS2 ade2-101/ADE2 abf1::LEU2/ABF1*	59
JCA11	*MATα leu2-3,112 ura3-52 his3Δ200 trp1Δ901 ade2-101 abf1::LEU2* YEp24-4c (YEp24 *ABF1 URA3*)	
JCA20	*MATα leu2-3,112 ura3-52 his3Δ200 trp1Δ901 ade2-101 abf1::LEU2* pR1190 (YCp19ΔNde *ABF1 TRP1*)	
JCA21	*MATα leu2-3,112 ura3-52 his3Δ200 trp1Δ901 ade2-101 abf1::LEU2* pR1191 (YCp19ΔNde *abf1-1 TRP1*)	
JCA22	*MATα leu2-3,112 ura3-52 his3Δ200 trp1Δ901 ade2-101 abf1::LEU2* pR1191 (YCp19ΔNde *abf1-2 TRP1*)	
JCA23	*MATα leu2-3,112 ura3-52 his3Δ200 trp1Δ901 ade2-101 abf1::LEU2* pR1193 (YCp19ΔNde *abf1-3 TRP1*)	
JCA24	*MATα leu2-3,112 ura3-52 his3Δ200 trp1Δ901 ade2-101 abf1::LEU2* pR1194 (YCp19ΔNde *abf1-4 TRP1*)	
JCA25	*MATα leu2-3,112 ura3-52 his3Δ200 trp1Δ901 ade2-101 abf1::LEU2* pR1195 (YCp19ΔNde *abf1-5 TRP1*)	
JCA26	*MATα leu2-3,112 ura3-52 his3Δ200 trp1Δ901 ade2-101 abf1::LEU2* pR1196 (YCp19ΔNde *abf1-6 TRP1*)	
YNN281	*MATa trp1Δ his3Δ200 ura3-52 lys2-801 ade2-1 gal*	Yeast Genetics Stock Center
YNN282	*MATα trp1Δ his3Δ200 ura3-52 lys2-801 ade2-1 gal*	Yeast Genetics Stock Center
JCA30	YNN281 *ABF1 HIS3*[a]	
JCA31	YNN281 *abf1-1 HIS3*[a]	
JCA32	YNN281 *abf1-2 HIS3*[a]	
JCA35	YNN281 *abf1-5 HIS3*[a]	
JCA40	YNN282 *ABF1 HIS3*[a]	
JCA41	YNN282 *abf1-1 HIS3*[a]	
JCA42	YNN282 *abf1-2 HIS3*[a]	
JCA45	YNN282 *abf1-5 HIS3*[a]	
AJ116	*MATa bar1-1 leu1 met trp5 ura3 can1*	A. Jong, University of Southern California
JCA50	*MATa bar1-1 ura3 lys2-801 met ABF1 HIS3*[a]	
JCA55	*MATa bar1-1 ura3 lys2-801 met abf1-5 HIS3*[a]	

[a] The *ABF1 HIS3* notation indicates that *HIS3* was integrated at the *ABF1* locus along with each *abf1* allele.

strain carrying a chromosomal disruption of the *abf1* gene and a wild-type *ABF1* gene on a *URA3* plasmid. Transformants carrying both plasmids were picked and screened for growth at 23 and 37°C to eliminate cells with temperature-sensitive mutations that are not recessive to the wild-type *ABF1* gene (i.e., unlinked or dominant lethal *ABF1* mutants). Transformants were also screened for growth at 23 and 37°C on plates containing 5-fluoroorotic acid to counterselect against the *URA3-ABF1* YEp24-4c plasmid. Of the 3,000 independent transformants picked, 9 gave temperature-sensitive growth and 49 (null mutations) failed to grow at 23 or 37°C on 5-fluoroorotic acid plates. When rescued from the temperature-sensitive strains into *E. coli* and retested by the plasmid shuffling protocol, six plasmids gave temperature-sensitive growth that was recessive to the wild-type *ABF1* gene. Three of the *abf1*(Ts) alleles were used to replace the chromosomal copy of the *ABF1* gene in a one-step gene replacement strategy described above.

To map the locations of the mutations responsible for the temperature-sensitive phenotype, we carried out marker rescue experiments as described by Budd et al. (12). Plasmids pMR1, pMR2, and pMR3 carrying different regions of the *ABF1* gene were constructed in the 2μm *ARS-URA3* vector pSEY18. pMR1 contains the *ABF1* 5′ flanking and coding region (1.1-kb *Hin*dIII-*Stu*I fragment), pMR2 contains the middle of the coding region (0.9-kb *Stu*I-*Kpn*I fragment), and pMR3 contains the 3′ coding and flanking region (1.25-kb *Kpn*I-*Spe*I fragment). Upon transformation into the *abf1*(Ts) strains, these plasmids were tested for the ability to rescue temperature-sensitive growth at 37°C via random recombination or gene conversion events. Once the region of the gene containing the *abf1*(Ts) mutation was defined, it was sequenced by the dideoxynucleotide method on double-stranded DNA with oligonucleotide primers specific to the *ABF1* gene.

Cell synchrony. A *MATa abf1-5 bar1* strain, JCA55, was constructed as described above and used to improve cell cycle arrest following treatment with α-factor. Cultures grown to early log phase (A_{600} = 0.3) at 25°C in YPD were treated with α-factor (0.3 μg/ml) for 6 h. By this time, 80 to 90% of the cells were unbudded. The growth medium was removed by filtration, and the cells were washed extensively with medium. The cells were resuspended in fresh medium to an A_{600} of 0.5 and incubated at 23 or 37°C. For incorporation time courses, cells were resuspended in synthetic medium, whereas cells analyzed by flow cytometry were resuspended in YPD. In reciprocal shift experiments, *abf1-5* cultures were incubated at the nonpermissive temperature and then treated with α-factor at the permissive temperature. These cells failed to progress synchronously through the cell cycle because of multiple cell cycle arrest points and slow recovery of the *abf1-5* mutant.

Macromolecular synthesis. In vivo levels of DNA and RNA synthesis were determined as described by Budd and Campbell (11), with slight modifications. Cells grown at 23°C in YPD were harvested in early log phase (A_{600} = 0.5) and resuspended in synthetic medium containing 7 μCi of [^{3}H] uracil per ml. The culture was split and incubated at 23 or 37°C for various times. For RNA synthesis determination, 0.1-ml aliquots were removed and precipitated with 10%

trichloroacetic acid (TCA)–0.1 M sodium pyrophosphate. The 0.4-ml aliquots taken for DNA synthesis determination were treated with NaOH to a final concentration of 1 M at 37°C for 24 h. The samples were neutralized to pH 7 with 0.1 M Tris · HCl, digested for 2 h at 37°C with 0.5 μg of RNase A per ml, and precipitated with 10% TCA–0.1 M sodium pyrophosphate. Protein synthesis rates were measured in 0.5-ml samples incubated with 10 μCi of Trans-^{35}S (ICN). The samples were precipitated with 10% TCA. All the samples were assayed in duplicate or triplicate, and each experiment was repeated at least three times.

DNA binding analysis. Yeast strains carrying the plasmid-borne *abf1*(Ts) alleles (100 ml) were grown to early log phase at 23°C, split, and incubated at 23 or 37°C for 3 h. The cells were harvested and yeast extracts were prepared as described previously (59). ABF1-DNA binding activity was measured by incubation with 20 μg of protein and 2 ng of ^{32}P-labeled *Hin*dIII-*Bgl*II fragment from YRp7 (*ARS1* domain B) in the presence of 3 μg of salmon sperm DNA as a nonspecific competitor for 15 min at 4°C. Gel retardation analysis was carried out at 25°C as described by Sweder et al. (68). To test in vitro DNA binding at the nonpermissive temperature, the binding reaction (10 min) and gel retardation were performed at 37°C. Western immunoblot analysis on 50 μg of the cell extract was carried out as described previously, using anti-ABF1 monoclonal antibody 6C11G4.

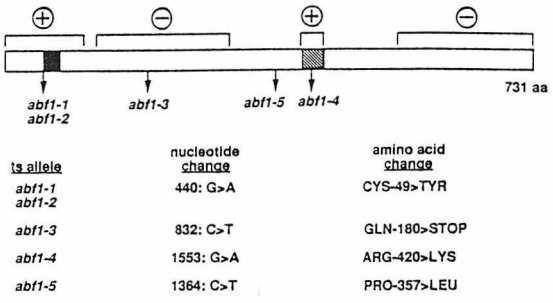
Mitotic plasmid stability assay. Single transformants were picked, grown under selective conditions overnight, diluted into nonselective YPD, and grown for 6 to 12 generations at 23 or 30°C. Aliquots of the initial and final culture were diluted, plated on YPD plates, and grown at 25°C. When colonies appeared, they were counted and replica spotted onto selective medium (medium minus uracil). The plasmid loss rate per generation was determined by $1-(F/I)^{1/N}$, where I is the initial percentage of plasmid-containing cells and F is the percentage of plasmid-containing cells after N generations of growth in nonselective medium. Plasmid loss determinations were made on at least four independent transformants for each plasmid.

β-Galactosidase assays. Yeast extracts prepared from 5 ml of early-log-phase cultures were assayed for β-galactosidase as described by Harshman et al. (37). Protein concentrations were determined by the Bradford assay. Specific activity of β-galactosidase was expressed as picomoles of *o*-nitrophenol produced per minute per milligram of protein. Measurements were made on at least four independent transformants per plasmid.

Flow cytometry. Samples collected for cell cycle analysis were fixed with 70% ethanol overnight at 4°C. The cells were pelleted and washed with 0.2 ml of Tris-buffered saline (50 mM Tris · HCl [pH 7.5], 150 mM NaCl). Following digestion in 0.5 mg of RNase A per ml for 2 h at 37°C, the cells were pelleted and resuspended in 0.3 ml of 100-μg/ml propidium iodide. The cells were briefly sonicated to break up aggregates, diluted to 10^6 cells per ml in Tris-buffered saline, and analyzed on an Ortho Cytofluorograf 50H-2150.

RESULTS

Isolation and characterization of *abf1*(Ts) mutants. A plasmid shuffling strategy was used to obtain temperature-sensitive mutations in the *ABF1* gene by introducing mutagenized copies of a plasmid-borne *ABF1* gene into JCA11 cells carrying a disruption in the essential chromosomal copy. Of the 3,000 colonies screened, nine mutants were identified that confer temperature-sensitive growth. By repeating the plasmid shuffling technique on plasmids recovered from these mutants, we found that six mutations were plasmid linked and recessive to the wild-type *ABF1* gene. Marker rescue experiments were carried out to define the regions of the *ABF1* gene responsible for the temperature-sensitive phenotype. DNA sequence analysis identified single-point mutations for five of the *abf1*(Ts) alleles (Fig. 1). In each case, the mutations were C-to-T transitions, as expected for hydroxylamine mutagenesis.



ts allele	nucleotide change	amino acid change
abf1-1 *abf1-2*	440: G>A	CYS-49>TYR
abf1-3	832: C>T	GLN-180>STOP
abf1-4	1553: G>A	ARG-420>LYS
abf1-5	1364: C>T	PRO-357>LEU

FIG. 1. Allele map of *abf1*(Ts) mutants. The bar represents the open reading frame of the *ABF1* gene and the relative positions of the *abf1*(Ts) mutations. The solid block represents the putative metal binding motif; the striped block represents the basic region. At the top are the relative regions of charge distribution across this highly acidic (net charge at pH 7 = −60) protein. The nucleotide and corresponding amino acid (aa) changes are shown. The positions of the nucleotides and amino acids are based on sequence analysis described in Rhode et al. (59).

The five mutations mapped to date fall within the N-terminal two-thirds of the protein. This region has been shown to be required for DNA binding activity (34; see below). Two of the mutations (*abf1-1* and *abf1-2*) were identical changes at nucleotide 440 resulting in a change of cysteine at position 49 to tyrosine. This residue falls within a region of the protein, C-49–X_7-H-57–X_3-H-61–X_4-C-66–X_4-C-71, that loosely resembles a metal binding motif (25, 34, 59). Previous mutational analysis showed that substitutions at H-57 or C-71 resulted in a mutant protein that, when isolated from *E. coli*, could no longer specifically bind DNA with high affinity (34). The mutation at nucleotide 1553 in the *abf1-4* allele falls in a basic region of the protein. This mutation results in a conservative arginine-to-lysine substitution at position 420. The mutation producing the temperature-sensitive phenotype of the *abf1-3* allele gives rise to a termination codon at position 180. We believe that this termination codon is suppressed in this strain, since extracts from this mutant still contain full-length ABF1 protein, as inferred from Western analysis (data not shown) and gel retardation analysis (see Fig. 3). The fifth mutation, *abf1-5*, is a proline-to-leucine change at amino acid 357 and therefore likely affects the structure of ABF1 rather than a specific active site, such as a DNA binding site.

Because of the high reversion rate of the sixth allele, *abf1-6*, marker rescue experiments proved inconclusive. However, sequence analysis of this allele failed to reveal any changes in the coding sequence.

DNA binding in *abf1*(Ts) mutants. Since five of the temperature-sensitive mutations mapped to a broad region of ABF1 important for DNA binding, we were interested in whether DNA binding was affected in the mutant proteins. Cell extracts were prepared from the *abf1*(Ts) mutants

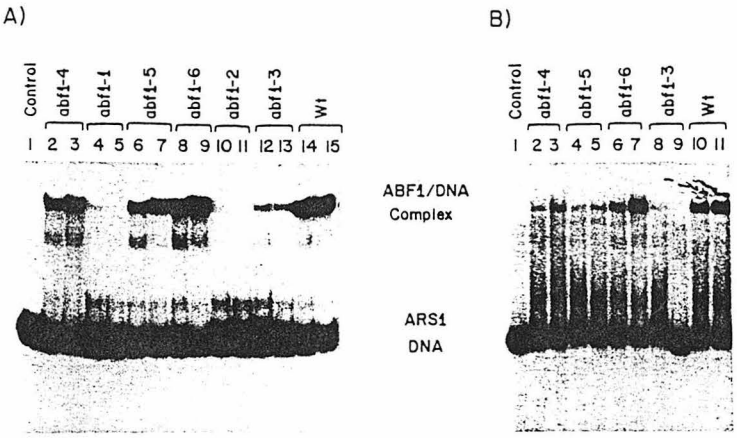


FIG. 2. DNA binding activities of *abf1*(Ts) proteins. Extracts from *abf1*(Ts) strains were prepared as described previously (59). Gel retardation analysis was used to detect ABF1 binding activity to a labeled DNA fragment containing the ABF1 binding site on domain B of *ARS1* as described in Materials and Methods. (A) Standard assay conditions (binding at 0°C for 15 min, gel run at 25°C) were used. Lane 1 contains no protein; lanes 2, 4, 6, 8, 10, 12, and 14 contain 20 μg of protein from cells grown at 23°C; lanes 3, 5, 7, 9, 11, 13, and 15 contain 20 μg of protein from cells shifted to 37°C for 4 h. Positions of the free *ARS1* DNA probe and ABF1-DNA complexes are indicated. (B) Gel retardation assays were carried out at the nonpermissive temperature (binding at 37°C for 10 min, gel run at 37°C) on the samples that showed ABF1 binding activity under standard conditions. Lane 1 contains no protein; lanes 2, 4, 6, 8, and 10 contain 20 μg of protein from cells grown at 23°C; lanes 3, 5, 7, 9, and 11 contain 20 μg of protein from cells shifted to 37°C for 4 h. Wt, wild type.

maintained at permissive and nonpermissive temperatures and were used in gel retardation assays with an *ARS1* DNA probe. When standard assay conditions were used, ABF1-DNA binding complexes were observed in extracts from *abf1-3*, *abf1-4*, *abf1-5*, and *abf1-6* strains, but little or no ABF1 binding activity was observed in the *abf1-1* (or *abf1-2*) extracts (Fig. 2A). A protein-DNA complex of higher mobility than the ABF1-DNA complexes was observed in extracts from *abf1-1* strains. However, competitive binding studies indicated that the faster-migrating complexes were not specific to the ABF1 binding site (59). The absence of any binding species in the *abf1-1,2* mutant makes it unlikely that there are other abundant ABF1-like binding activities present in these extracts. The absence of ABF1 binding activity in the *abf1-1* strain was not due to reduced protein levels. Western analysis indicated that wild-type levels of the ABF1 protein doublet were observed in each of the mutants except the *abf1-3* strain, which had about 20% of wild-type levels of ABF1 (data not shown). For each mutant, only slight differences were seen between the level of binding activity in extracts from cells maintained at either the permissive or nonpermissive temperature, suggesting that there is not rapid decay of the protein at high temperatures.

To determine whether any of the mutant proteins showed thermolabile binding, we performed gel retardation assays at 37°C (Fig. 2B). Under these assay conditions, ABF1 binding activity in extracts of the *abf1-3*, *abf1-4*, and *abf1-5* mutants was lower than that seen in extracts from the wild-type strain or in comparison with binding at 23°C (Fig. 2A), indicating thermolability of the mutant proteins. The lack of ABF1 binding activity in the *abf1-1* strain at all temperatures and the thermolability of this activity in the *abf1-3*, *abf1-4*, and *abf1-5* strains suggest that the mutations fall at residues that interact with the DNA or are important for the structural integrity of the DNA binding domain. The fact that reduced ABF1 binding activity was observed in vitro at the nonpermissive temperature for the *abf1-3* allele suggests that suppression of the nonsense mutation results in a thermolabile protein. The *abf1-6* mutant showed wild-type levels of ABF1 binding activity with no apparent temperature sensitivity, again consistent with the deduced location of the *abf1-6* lesion outside of the DNA binding domain.

Properties of *abf1*(Ts) mutants. To examine growth arrest of the *abf1*(Ts) mutants, we plated JCA11 cells carrying the various plasmid-borne *abf1*(Ts) alleles on solid media and incubated the cells at the nonpermissive temperature for 2 days. Three of the mutants (*abf1-1*, *abf1-2*, and *abf1-5*) showed little or no growth at 37°C, while the other mutants (*abf1-3*, *abf1-4*, and *abf1-6*) grew slowly, forming small colonies. The *abf1*(Ts) alleles that gave rapid growth arrest were used to replace the chromosomal copy of the *ABF1* gene as described in Materials and Methods. Growth of these mutants arrested in a single cell cycle at the nonpermissive temperature, as determined microscopically. As shown in Fig. 3, the arrested cells were enlarged and had small elongated buds (see legend to Fig. 3). Some buds contained DNA (not shown), and some did not. This phenotype is distinct from that of any known cell division cycle mutants that arrest at G_1/S, though it resembles that of cells overproducing STE12p, a transcription factor involved in pheromone-responsive gene expression (26). The complex appearance may be due to arrest at two points in the cycle (see also Fig. 5). The growth rate of the *abf1-1* mutants at the permissive temperature is nearly identical to that of the isogenic ABF1 strain, while the *abf1-5* strains grew more slowly. Both *abf1-1* and *abf1-5* strains grew slowly at 30°C but arrested growth at temperatures of ≥32°C.

ARS activity is reduced in *abf1*(Ts) strains. Mutational analysis of the ABF1 binding sites in *ARS1*, *ARS121*, and the *HMRE* ARS suggest that ABF1 plays an important role in ARS function. To determine directly the requirements of different ARSs for ABF1 function, we measured the ARS activity for three *ARS-CEN* plasmids (*ARS1*, *ARS121*, and

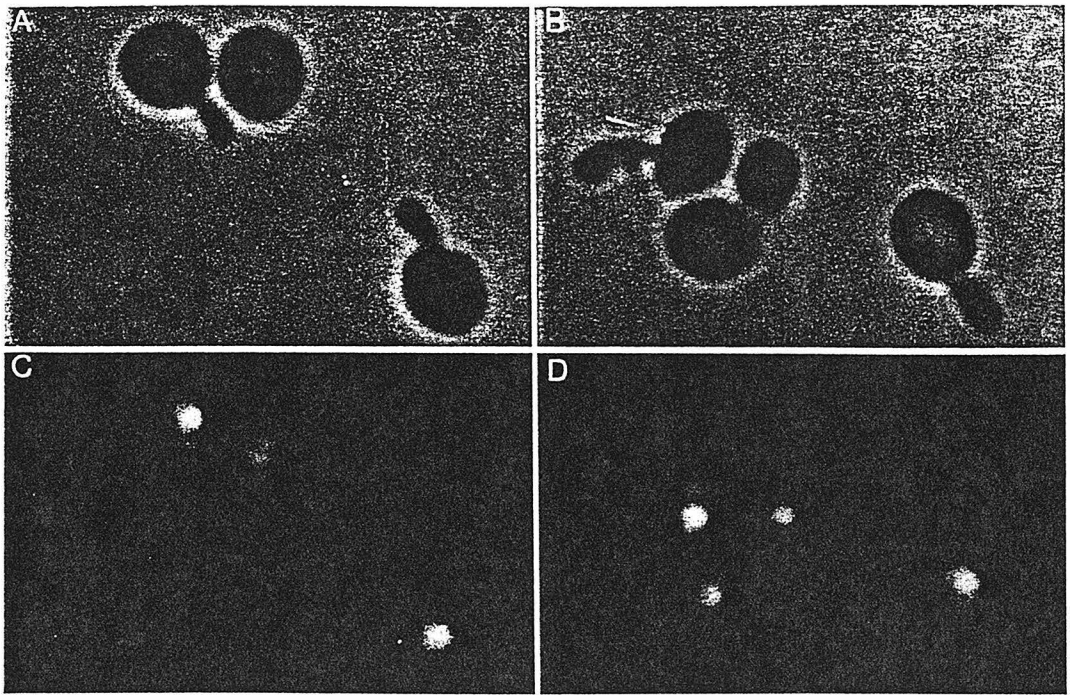


FIG. 3. Arrest phenotypes of *abf1-1* mutants. JCA41 (*abf1-1*) (A and C) and JCA45 (*abf1-5*) (B and D) strains were grown at 23°C to early log phase and then shifted to 37°C for 4 h. The cells were harvested and fixed with methanol-acetic acid (3:1), and the nuclei were stained with 4′,6-diamidino-2-phenylindole as described previously (43). (A and B) Phase-contrast field; (C and D) fluorescence field. Percentages of cells with elongated buds: *abf1-1*, 70%; *abf1-5*, 40%; *ABF1*, 0%.

H4 ARS) in *abf1*(Ts) strains maintained at permissive and semipermissive growth temperatures. Of the ARSs tested, *ARS121* and *ARS1* have well-characterized high-affinity ABF1 binding sites, such that purified ABF1 has a fivefold-higher affinity for *ARS121* than for *ARS1* (30). ABF1 fails to recognize the *H4* ARS with high affinity (8). Previous studies have also shown that *ARS121* has two ABF1 binding sites within domain B, which when deleted result in an increase in plasmid instability (as measured by plasmid loss per generation in nonselective conditions) from about 5% for the wild-type *ARS121* to about 12% for the domain B-deletion mutant (73, 74). Similarly, deletions into the single ABF1 binding site in domain B of *ARS1* result in an increase in plasmid loss rate from 10% for the wild-type ARS1 to 13% for the domain B-deletion mutant (24).

When the mitotic stability of these ARS plasmids was examined in the *abf1*(Ts) strains grown at the semipermissive temperature, *ARS121* and the *H4 ARS* plasmids showed

TABLE 2. ARS plasmid stability in *abf1*(Ts) strains

ARS plasmid	Strain	Semipermissive temp (30°C)				Permissive temp (25°C)			
		% Plasmid-containing cells		% Plasmid loss/generation[a]	Ratio[b]	% Plasmid-containing gels		% Plasmid loss/generation	Ratio
		Selective	Nonselective			Selective	Nonselective		
ARS121	*abf1-1*	75.0 ± 2.0	28.8 ± 6.9	17.5 ± 3.7[c]	26	77.5 ± 1.4	58.8 ± 3.8	2.5 ± 0.4	0.8
	abf1-5	68.8 ± 5.2	22.5 ± 8.5	13.2 ± 3.7[c]	20	68.8 ± 6.3	45.0 ± 6.5	4.1 ± 0.6	1.3
	ABF1	76.9 ± 4.0	73.8 ± 0.7	0.7 ± 0.3		80.0 ± 3.5	53.8 ± 3.8	3.1 ± 0.5	
ARS1	*abf1-1*	91.3 ± 4.3	72.5 ± 6.0	3.9 ± 1.5	1.5	92.5 ± 3.2	67.5 ± 3.2	2.5 ± 0.4	0.9
	abf1-5	86.3 ± 2.5	50.0 ± 10.0	6.5 ± 2.5	2.5	96.3 ± 2.5	68.8 ± 3.8	3.1 ± 0.5	1.1
	ABF1	86.3 ± 3.8	63.8 ± 6.6	2.6 ± 1.0		85.0 ± 3.8	62.5 ± 5.2	2.8 ± 0.5	
H4 *ARS*	*abf1-1*	88.8 ± 4.3	67.5 ± 1.4	4.3 ± 0.4[c]	2.4	93.1 ± 2.1	78.8 ± 5.5	1.3 ± 0.5	0.6
	abf1-5	85.0 ± 6.5	65.0 ± 4.6	3.6 ± 0.6[c]	2.0	93.8 ± 3.8	75.0 ± 4.1	1.9 ± 0.4	1.0
	ABF1	93.8 ± 2.4	76.3 ± 4.7	1.8 ± 0.5		93.8 ± 1.3	73.8 ± 3.1	2.0 ± 0.3	

[a] Determined as $1-(F/I)^{1/N}$, where I is the initial percentage of plasmid-containing cells and F is the percentage of plasmid-containing cells after N generations of growth in nonselective medium.

[b] Ratio of percent plasmid loss per generation of the *abf1*(Ts) strain to that of wild-type strain.

[c] Percent plasmid loss per generation in the *abf1*(Ts) mutant is significantly different ($P < 0.02$) from the loss rate in the wild-type strains.

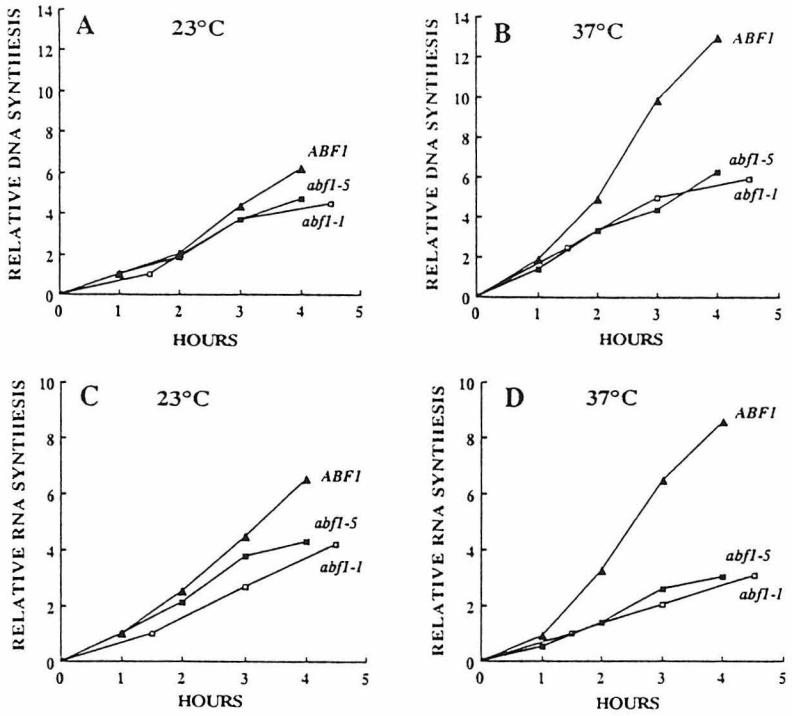


FIG. 4. DNA and RNA synthesis in *abf1*(Ts) mutant strains. [^{3}H]uracil incorporation (7 μCi/ml) into DNA and RNA was determined as described in Materials and Methods. To compare incorporation rates between strains, the level of incorporation (counts per minute per milliliter) for any given time was normalized to that seen after 1 h at 23°C. The relative rates of DNA synthesis at 23°C (A) and 37°C (B) are shown for asynchronous JCA41 (*abf1-1*), JCA45 (*abf1-5*) and JCA40 (*ABF1*) strains. Similarly, relative RNA synthesis rates for these strains are shown for cultures grown at 23°C (C) and 37°C (D). Each point represents the average of duplicate determinations.

a statistically significant decrease in stability compared with loss rates measured in the wild-type strain (Table 2). Furthermore, differential effects were observed depending on the ARS element, indicating that this phenotype was not merely the result of a plasmid segregation defect or poor cell growth. In both *abf1-1* and *abf1-5* mutant strains, *ARS121* plasmids were lost at a rate at least 20-fold greater than that seen in wild-type cells. *H4 ARS* plasmids were lost at about 2-fold-greater rates in the *abf1*(Ts) mutants than in wild-type cells, while loss rates for *ARS1* plasmids were 1.5- to 2.5-fold greater than wild-type rates in the two *abf1*(Ts) strains, respectively. This loss of stability was temperature dependent, since *abf1-1*, *abf1-5*, and wild-type strains showed little or no difference in plasmid loss rates at the permissive temperature (Table 2). These results provide evidence for a direct role for the *abf1*(Ts) protein in ARS activity. Furthermore, the magnitude of the effect of the *abf1*(Ts) alleles on *ARS1* and *ARS121* plasmid loss rates is comparable to the magnitude of the effect of ABF1 binding site mutations on ARS activity summarized above.

Effects of the *abf1*(Ts) alleles on DNA and RNA synthesis in asynchronously growing cultures. The morphology of the *abf1*(Ts) mutants arrested at 37°C (Fig. 3) gave no indication of a specific effect on cell cycle progression or DNA replication but suggested that ABF1 may be required at multiple points in the cell cycle. To examine the effect of ABF1 on cell growth in more detail, we measured DNA and RNA synthesis in the *abf1*(Ts) strains during incubation at the nonpermissive growth temperature. The strains were grown to early log phase at the permissive temperature, and the cultures were divided and incubated at permissive and nonpermissive temperatures in the presence of [^{3}H]uracil. At various times, aliquots were removed and nucleotide incorporation into DNA and RNA was determined. At the permissive temperature, DNA and RNA synthesis rates were only slightly lower in the *abf1-1* and *abf1-5* cultures than in the wild-type culture (Fig. 4A and C). In contrast, after 1 h at 37°C, the extent of DNA synthesis in both *abf1-1* and *abf1-5* strains was reduced to 50% of that seen in the wild-type strain and total RNA synthesis in these cultures was reduced to about 40% of wild-type levels (Fig. 4B and D). These findings are consistent with multiple roles for ABF1 documented from earlier studies involving mutagenesis of *cis*-acting DNA binding sites (see Discussion). The defect in RNA synthesis may represent a more general role of ABF1 in transcription than previously appreciated from the study of individual genes.

Although DNA synthesis at 37°C was lower in the *abf1*(Ts) strains than in the wild type (Fig. 4B), it was not completely defective. In fact, DNA synthesis in the *abf1*(Ts) strains continued for several hours at the same rate as was seen in cells grown at the permissive temperature, even though no division was observed for up to 6 h at the nonpermissive temperature. These results have two possible explanations.

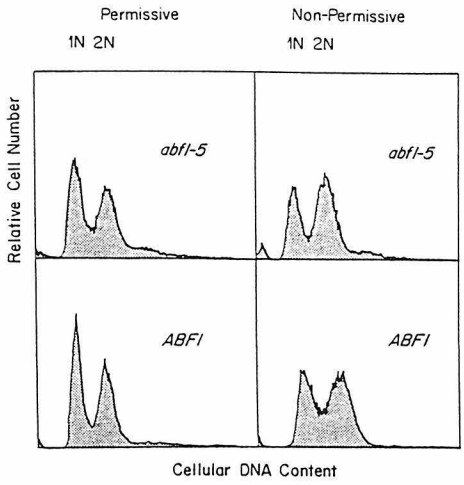


FIG. 5. Flow cytometric analysis of *abf1-5* cells. JCA45 (*abf1-5*) cells were grown to early log phase at 23°C and shifted to 37°C for 4 h. The cells were then harvested and fixed with 70% ethanol, and cellular DNA was stained with propidium iodide as described in Materials and Methods. Shown are DNA histograms of the propidium iodide-stained cells determined by flow cytometry.

The less than wild-type level of ongoing synthesis at 37°C could be due to reduced rates in all cells; alternatively, some cells could complete replication of replicons already initiated at the time of the shift-up and arrest in G_2, while other cells in the population fail to initiate any DNA synthesis and arrest in G_1 phase. The latter interpretation is supported by the results of flow cytometric analysis. As shown in Fig. 5, *abf1-5* cells arrested at 37°C consisted of two discrete populations, one in G_1 and one in G_2. A more direct test of the effect on initiation versus elongation of DNA synthesis is discussed below.

Effects of the *abf1*(Ts) alleles in synchronously growing cultures. To examine whether the initiation of DNA replication was affected in the *abf1*(Ts) mutants, DNA synthesis and cell cycle progression were monitored in cells synchronized with α-factor. An *abf1-5 bar1* culture incubated at the permissive temperature was treated with α-factor until growth arrested at START. (The *bar1* mutant was used in these studies to reduce the amount of α-factor needed and thus prevent nonspecific toxicity.) The α-factor was removed, and the cells were shifted to the nonpermissive temperature in media containing [^{3}H]uracil. DNA synthesis rates in these cultures were found to be 50% of that seen in cultures released from α-factor arrest at the permissive temperature (Fig. 6A). Furthermore, the magnitude of this defect is even greater when compared with DNA synthesis in a synchronized *ABF1* strain, in which the incorporation

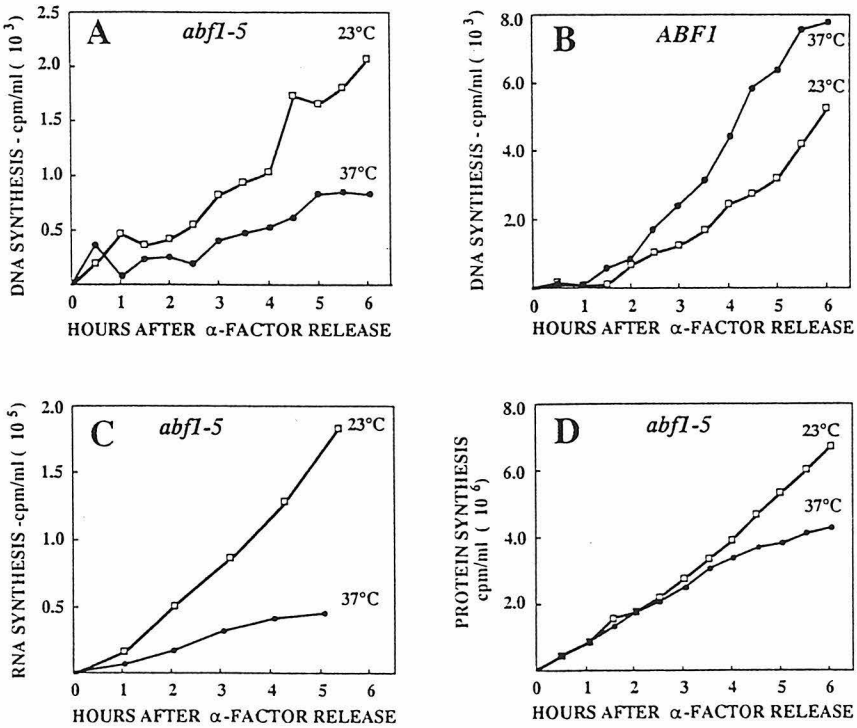


FIG. 6. DNA, RNA, and protein synthesis in α-factor-synchronized *abf1-5* cells. JCA55 (*bar1-1 abf1-5*) and JCA50 (*bar1-1 ABF1*) strains were arrested at START by treatment with 0.3 μg of α-factor per ml as described in Materials and Methods. Following the removal of α-factor, macromolecular synthesis was measured in cells incubated at 23°C (□) or 37°C (●). The time courses of [^{3}H]uracil incorporation into DNA are shown for JCA55 (A) and JCA50 (B) cells. [^{3}H]uracil incorporation into RNA (C) and Trans-^{35}S (methionine and cysteine) incorporation into protein (D) are shown for JCA55 cells. Each point represents the average of duplicate determinations.

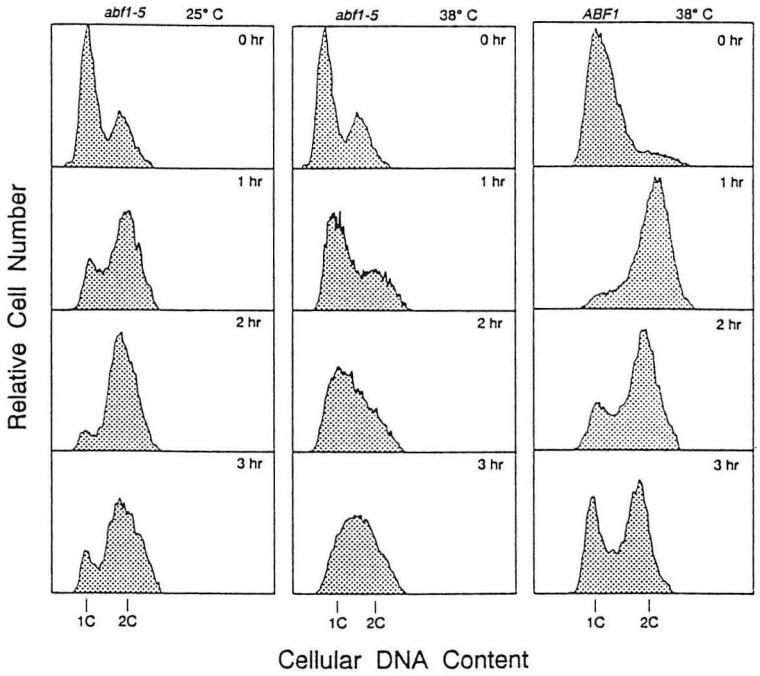


FIG. 7. Cell cycle progression of α-factor-synchronized *abf1-5* cells. JCA55 (*bar1-1 abf1-5*) and JCA50 (*bar1-1 ABF1*) strains were arrested with α-factor as described in Materials and Methods. Following the removal of α-factor, the cells were incubated at 25 or 38°C in YPD. At the indicated times, aliquots were removed and fixed with 70% ethanol, and cellular DNA was stained with propidium iodide as described in Materials and Methods. Shown are DNA histograms of the propidium iodide-stained cells determined by flow cytometry.

rate at 37°C was twice that seen at 23°C (Fig. 6B). Cells that were arrested with α-factor, released at the nonpermissive temperature for 1 or 2 h, and shifted back to the permissive temperature recovered the ability to synthesize DNA at the same rate as was observed in cells initially released at the permissive temperature (data not shown), indicating that the replication defect was reversible and cannot be attributable to a loss of cell viability. By comparison with other mutants involved in DNA replication, this defect was greater than the DNA replication defect of a *cdc2* mutant, which carries a defective DNA polymerase δ (20).

Similar incorporation time courses were carried out to measure RNA and protein synthesis levels in synchronized *abf1-5* cells at the nonpermissive temperature. In these cultures, RNA synthesis was inhibited to 35% of the level seen at the permissive temperature (Fig. 6C), consistent with the effects seen in asynchronous cultures. In contrast, protein synthesis rates were identical for several hours in the synchronized *abf1-5* cultures incubated at nonpermissive and permissive temperatures (Fig. 6D). As was observed for DNA synthesis (Fig. 6B), synchronized *ABF1* cells released at 37°C synthesized RNA and protein at approximately twice the rate measured at 23°C (data not shown).

Because RNA and to a lesser extent protein synthesis are affected in *abf1-5* cultures at the nonpermissive temperature, it is difficult to make conclusions as to the direct role of ABF1 in DNA replication. However, the extent and reversibility of the DNA synthesis defect suggest that some or all of these cells fail to initiate and progress through S phase efficiently as the result of the loss of ABF1 function. This hypothesis was supported by flow cytometric analysis of *abf1-5* cells synchronized with α-factor and then released at either the permissive or nonpermissive temperature. Figure 7 shows that α-factor treatment resulted in arrest of approximately 70% of the *abf1-5* cells at START with a 1C content of DNA. Following the removal of α-factor, cultures incubated at the permissive temperature for 2 h had progressed through S phase and into G_2 (2C DNA content). Cultures incubated at the nonpermissive temperature recovered from α-factor arrest but initiated and progressed through S phase inefficiently, consistent with the defect in DNA synthesis seen in Fig. 6. As a result, by 3 h after release from α-factor arrest, less than half of the G_1 cells showed an increase in DNA content. A precise estimate is not possible because of broadening of the G_1 and G_2 peaks for *abf1*(Ts) cells arrested at the nonpermissive temperature (data not shown). The isogenic *ABF1 bar1* strain synchronized in the same manner progressed efficiently through the cell cycle at both 25°C (not shown) and 38°C (Fig. 7).

Transcriptional activation function of the ABF1 binding site is defective in an *abf1-1* strain. Sequence data base searches have revealed that as many as 80 yeast genes contain ABF1 binding sites within their promoters. Several laboratories have shown by mutational analysis that these sites play an important role in both positive and negative regulation of gene expression (4, 6, 9, 16, 21, 22, 27, 35, 36, 38, 47). Furthermore, when the ABF1 binding site from the *HMRE* silencer was inserted into a promoter lacking a functional UAS, fourfold transcriptional activation was observed (4, 9). However, when UAS activity was determined for ABF1

TABLE 3. UAS activity in an *abf1-1* strain

UAS element	Strain	Semipermissive temp (30°C)		Permissive temp (25°C)	
		β-Galactosidase sp act (U/mg)	UAS activity[a]	β-Galactosidase sp act (U/mg)	UAS activity
None	*abf1-1*	1.65 ± 0.09	1	0.60 ± 0.02	1
	ABF1	1.42 ± 0.05	1	0.58 ± 0.05	1
ABS	*abf1-1*	1.82 ± 0.04	1.1	1.88 ± 0.15	3.1
	ABF1	4.97 ± 0.25	3.5	3.16 ± 0.19	5.4
ARE	*abf1-1*	319 ± 30	194	445 ± 11	734
	ABF1	453 ± 9	320	431 ± 56	737

[a] Ratio of β-galactosidase activity in a strain carrying the *UAS-cyc1-lacZ* construct versus the pLGΔB (no UAS) plasmid.

binding sites that had a 20-fold range in affinity for purified ABF1, no correlation between the ABF1 relative binding affinity in vitro and UAS activity of these sites was observed (9). Therefore, it is not clear whether UAS function was due to the direct action of ABF1 or to other factors that might bind to these sites. To address the role of ABF1 in transcriptional activation, we tested the UAS function of the ABF1 binding site in an *abf1-1* strain. An oligonucleotide (designated ABS) corresponding to the ABF1 binding site at *ARS1* was inserted into the UAS-less *CYC1* promoter-*lacZ* fusion gene of the pLGΔB plasmid. This plasmid was used to transform *abf1-1* and wild-type strains, and transformants maintained at permissive and semipermissive temperatures were assayed for β-galactosidase activity (Table 3). Basal levels of β-galactosidase activity were identical in *abf1-1* and wild-type strains carrying the pLGΔB plasmid lacking the UAS. When the ABS oligonucleotide was inserted in this plasmid, a three- to fivefold increase in β-galactosidase activity was observed in both the *abf1-1* and wild-type strains maintained at 25°C, consistent with the weak UAS activity of the ABF1 binding site reported previously. However, the ABF1 binding site failed to activate transcription above basal levels in the *abf1-1* strain grown at the semipermissive temperature. Wild-type cells showed a threefold increase in β-galactosidase activity under these semipermissive conditions.

ABF1 binding sites are found in the promoters of genes encoding subunits of RNA polymerases, translational initiation factors, and ribosomal proteins (21, 22). Thus, the *abf1-1* allele may be affecting β-galactosidase activity indirectly by altering levels of the transcriptional or translational machinery. Additional effects of the pLGΔB plasmid levels also may result in lower β-galactosidase activity in this strain. To address whether the *abf1-1* allele is indirectly affecting induction of β-galactosidase activity at the semipermissive temperature, we assayed the UAS activity of the binding site of the yeast transcription factor yAP1. Previous studies have shown that this site, called the ARE oligonucleotide, acts as a strong UAS element and that yAP1 activates ARE-*CYC1*:*lacZ* reporter gene expression in a temperature-independent manner (37, 54). Unlike the ABF1 binding site, the ARE oligonucleotide acted as a strong UAS in the *abf1-1* strain at the semipermissive temperature, increasing β-galactosidase activity 200-fold over basal levels. However, ARE-UAS function was reduced to 60% of that seen in the wild-type strain, suggesting some ABS-independent effects of the *abf1-1* allele on full induction of β-galactosidase activity. This effect was not observed at the permissive temperature, at which ARE-UAS function was equivalent in the *abf1-1* and wild-type strains. These results suggest that the complete temperature-dependent loss of ABS-UAS function in the *abf1-1* strain is due at least in part to the action of ABF1 on transcriptional activation at this site.

Mapping of essential regions of ABF1 by using *abf1*(Ts) alleles. To begin to address structure-function relationships for ABF1, we cloned a series of 3′ deletions of the ABF1 coding sequence into an *ARS-CEN-URA3* plasmid and introduced these constructs into the *abf1-1* strain. Transformants were used to determine whether C-terminal regions of ABF1 were required for DNA binding activity and for complementation of the *abf1-1* temperature-sensitive growth phenotype. As shown in Fig. 2A, extracts from *abf1-1* strains grown at the permissive temperature did not contain measurable ABF1 binding activity. When a full-length *ABF1* gene (construct H2.6) or a 500-bp deletion in the 3′ end of *ABF1* (construct H2.1) was introduced into this strain, extracts showed ABF1-specific DNA binding activity, whereas no DNA binding was observed when deletions of 1 kb (or larger) from the 3′ end of *ABF1* were tested (Fig. 8). These results indicate that the C-terminal region of the protein is dispensable for DNA binding activity in vitro and are consistent with the findings of similar DNA binding

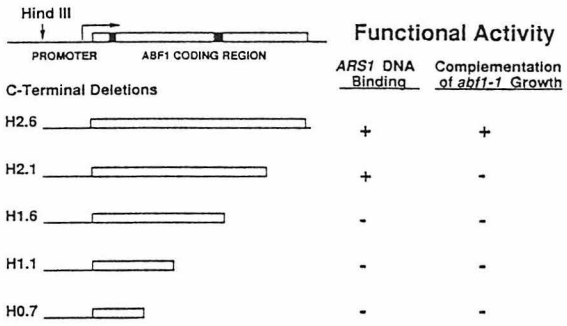


FIG. 8. Structure-function analysis of the *ABF1* gene. A series of *URA3-ARS-CEN* plasmids bearing 3′ deletions in the *ABF1* gene was constructed as described in Materials and Methods. The bars represent the *ABF1* coding region for the intact and deletion constructs. The solid block represents the putative metal binding motif; the striped block represents the basic region described in the text. The normal *ABF1* promoter and transcriptional termination sequences are at the 5′ and 3′ ends, respectively, of the deleted *ABF1* genes. Following introduction into JCA31 (*abf1-1 ura3-52*) cells, the deletion constructs were tested for the ability to bind an oligonucleotide (element B) for the ABF1 binding site at *ARS1* as assayed by gel retardation (59) and for complementation of the *abf1-1* growth defect at 32°C.

studies on bacterially produced ABF1 (34). None of the C-terminal deletion constructs were able to complement the growth defect of the *abf1-1* strain at 32°C (the minimum nonpermissive temperature), indicating that ABF1 DNA binding activity alone is not sufficient to provide all of the essential functions mediated by ABF1 in vivo.

DISCUSSION

Mutational analysis of several ARSs previously suggested a role for ABF1 in DNA replication by defining the ABF1 binding site as a *cis*-acting element important though not essential for ARS activity. For example, Diffley and Stillman (24) showed that deletion of the ABF1 binding site at *ARS1* resulted in a small but detectable decrease in plasmid stability. This defect was exaggerated when similar deletions were assayed under suboptimal growth conditions (galactose as a carbon source) (69). Mutagenesis of the ABF1 binding site at the *HMRE* ARS gave more ambiguous results. In the case of a deletion, a large defect in plasmid stability was observed (3), while a point mutation in the ABF1 binding site showed no detectable effects (47). Linker-scanning mutations in domain B of *ARS121* that removed the ABF1 binding site also resulted in a threefold decrease in plasmid stability (74). In addition, the ABF1 binding site has been shown to act as an orientation- and position-independent enhancer of ARS activity at *ARS121* (73). It is intriguing that the degree of dependence of ARS activity on the ABF1 binding site correlated with ABF1 binding affinity at these ARSs. For instance, purified ABF1 has a 5- to 10-fold higher affinity for *ARS121* than for *ARS1* or the *HMRE* ARS (30), and ABF1 binding site mutations have a more deleterious effect on *ARS121* than on *ARS1*. These findings further imply that occupancy of the ABF1 binding site is important for ARS activity.

Based on the temperature-dependent defect in *ARS-CEN* plasmid stability, we have demonstrated a role of ABF1 on ARS activity in the *abf1*(Ts) strains. The magnitude of this defect for *ARS1* and *ARS121* plasmids correlated with the effect of ABF1 binding site mutations at these ARSs reported previously. The stability of the *H4 ARS* plasmid was also slightly defective in the *abf1*(Ts) strains. High-affinity ABF1 binding sites have not been reported for this ARS, suggesting that the small defect in plasmid stability observed may be an indirect effect or mediated through low-affinity ABF1 binding sites. Several other functional ARSs, including the *H2B* ARS and the *HMLE* ARS, apparently lack high-affinity ABF1 binding sites (8). Thus, it seems likely that the requirement for ABF1 can be fulfilled by other *cis*-acting elements at these ARSs and (under optimal growth conditions) at *ARS1*.

Minichromosome maintenance-defective (*mcm*) mutants representing 18 different complementation groups that result in decreased stability of *ARS-CEN* plasmids have been isolated (32, 50). Like the *abf1*(Ts) mutants, several of these mutants (*mcm1*, *mcm2*, and *mcm3*) showed an ARS-specific defect, suggesting a role in plasmid replication rather than segregation or other functions required for stability (32). However, the *abf1*(Ts) mutants clearly have an ARS specificity different from that of the *mcm1*, *mcm2*, or *mcm3* mutants. In fact, *ARS121* plasmids are unstable while *ARS1* plasmids are stable in the *abf1*(Ts) strain at the nonpermissive temperature, whereas exactly the opposite pattern of plasmid stability is observed in the *mcm* mutants. Although direct roles of the *mcm* mutants in ARS function remain to be demonstrated, these findings suggest that ABF1 and the MCM proteins may act independently through different functional elements at different ARSs.

Like ABF1, MCM1 is a transcription factor that is involved in activation or repression of transcription (1, 46, 57). At least one other well-defined transcription factor has been implicated in ARS function as a result of a search for mutations that increased the stability of plasmids that carried the *HO* ARS, in which a point mutation caused increased plasmid loss (48). Of three genes identified, *RAR1*, *RAR3*, and *RAR5*, *RAR3* has been shown to encode a protein identical to the GAL11/SPT13 transcription factor (48). GAL11/SPT13 has been shown to be a general transcription factor that acts at a large number of genes and to have both activator and repressor function. Thus, at least two transcription factors in addition to ABF1 have been shown to affect ARS function, at least as measured on plasmids, and a picture of ARS organization in which a core activating sequence is influenced by association with different enhancers is emerging.

While the roles of ABF1 binding sites and mutations affecting them have been evaluated on *ARS* plasmids, the effects of ABF1 site mutations on origins of replication in their chromosomal location has not yet been investigated by two-dimensional gel analysis. However, evaluation of DNA replication in *abf1*(Ts) mutants suggested that ABF1 plays a role in the initiation of chromosomal DNA replication. Conclusions as to whether this is a direct role are limited by the fact that the *abf1-5* mutations also affect transcription in these strains. Synchronized *abf1-5* cultures recovered from α-factor arrest and initiated DNA synthesis at the same time as did wild-type cells (Fig. 6) but failed to progress through S phase (Fig. 7) and showed reduced rates of DNA synthesis at the nonpermissive temperature. This defect was reversible when the *abf1-5* cultures were shifted down to the permissive temperature. The residual DNA synthesis observed in the *abf1-5* strain may be due to initiation at replication origins not dependent on ABF1, mitochondrial DNA synthesis, or leakiness of this allele. Comparison of DNA synthesis rates in the asynchronous *abf1*(Ts) cultures suggests that ongoing DNA synthesis can continue in these cells at the nonpermissive temperature. Furthermore, when synchronized *abf1-5* cells were allowed to progress into S phase for 1 or 2 h and then shifted to the nonpermissive temperature, rates of DNA synthesis and cell cycle progression were intermediate to those seen at the permissive and nonpermissive temperatures (58). These results may indicate that DNA synthesis initiated prior to the temperature shift can continue either at a slightly slower rate or at the same rate while further initiation at late-replicating origins is inhibited; alternatively, some cells may fail to initiate altogether, depending on the stage of the cell cycle when the nonpermissive condition is introduced. The initiation defect at 37°C in the *abf1-5* mutant appears to be greater than expected on the basis of the variable requirement for ABF1 at ARS elements observed in the plasmid stability assays. The necessity of carrying out the ARS stability assays at the semipermissive temperature may account for this finding. Alternatively, ABF1 has greater or lesser importance at each ARS, depending on the other proteins that interact there. It will be of great interest to examine the sequence requirements for chromosomal origins of replication as well as origin utilization in an *abf1*(Ts) strain.

It has been proposed that ABF1 plays a role in the transcriptional activation or repression of as many as 80 genes. Given that there are five ABF1 binding sites in the promoter of the *ABF1* gene, this protein may even regulate

its own expression (34, 58). We found that the ability of the ABF1 binding site to act as a UAS element in the *CYC1-lacZ* test plasmid was completely temperature sensitive in an *abf1-1* strain, demonstrating a direct role of ABF1 in transcriptional activation. The specific effect of the *abf1*(Ts) alleles on the transcription of yeast genes that ABF1 is thought to control, including repression of the silent mating-type loci, is currently under investigation. Recently, Kurtz and Shore (49) used a similar approach to demonstrate that RAP1 regulates transcriptional activation of *MATα* genes and transcriptional repression at *HMR*. In addition to the effects on specific transcriptional activation, we observed a significant defect in general RNA synthesis in the *abf1*(Ts) strains at the nonpermissive temperature. This defect may be the result of an ABF1 function directly at the 35S rRNA gene. ABF1 is likely to interact with a protein recognition site (the REB2 site) found in the transcriptional enhancer/terminator element of the rRNA repeat (17, 53). However, the functional role of this site in enhancer activity has yet to be investigated. Alternatively, since ABF1 binding sites are found in the promoters of genes encoding subunits of RNA polymerases I and III (22), it is possible that the defect in RNA synthesis is due to a reduction in RNA polymerase activity. ABF1 also may affect transcription of genes that control translation, including those encoding ribosomal proteins, translational initiation factors, and aminoacyl-tRNA synthetases (see reference 21 for references); however, little or no decrease in protein synthesis rates was observed in the *abf1*(Ts) mutants at the nonpermissive temperature.

How a single DNA-binding protein can regulate a number of important nuclear processes is currently of great interest. As mentioned above, ABF1 is functionally similar to RAP1 and MCM1. These proteins contribute to repression or activation of transcription, depending on the context of their binding sites (1, 10, 46, 57, 61). In addition, MCM1 may function in the initiation of DNA replication at a subset of ARS elements (57), and RAP1 binds to and controls the length of poly($C_{1-3}A$) tracts at telomeres (10, 50). While it remains possible that these factors act through some general mechanism such as nucleosome exclusion or DNA bending at functionally distinct sites, it appears most likely that they mediate various functions by specific combinatorial interactions with other structural or regulatory proteins in a context-dependent manner. In support of this hypothesis, recent mutational analysis indicated that different domains of these proteins are required for different functions (19, 49). For example, the DNA binding domain of MCM1 is sufficient for this protein's essential function, the minichromosome maintenance phenotype, and transcriptional repression of α-specific genes (19). Additional domains are required for transcriptional regulation of α-specific genes. Unlike MCM1, structure-function studies of the *ABF1* gene clearly demonstrate that the DNA binding domain alone cannot perform all of ABF1's essential functions. Through complementation of the phenotypes observed in the *abf1*(Ts) mutants, we hope to further define the domains of ABF1 responsible for ARS function and transcriptional control. It is also possible that ABF1 exists in several functionally distinct states. We have recently found that ABF1 is a phosphoprotein with at least four different forms (64). ABF1 phosphorylation states vary with different growth conditions and correlate with the expression of ABF1-regulated genes such as *COX6*. In addition, ABF1 containing protein-*COX6* promoter DNA complexes have different electrophoretic mobilities, depending of the phosphorylation state of ABF1. These results suggest that interaction with other factors may be dependent on the phosphorylated state of ABF1 (64).

Several viral and mammalian transcription factors have been found to activate viral DNA replication through interactions at auxiliary binding sites in the viral origin (see reference 23 for a review). These proteins appear to function in DNA replication by a variety of mechanisms, including nucleosome exclusion from the replication origin (18), facilitated interactions of the initiator protein with its binding site (52), and activation of the DNA unwinding activity of the initiator (33). At the *ARS1* origin, the ABF1 binding site is found at the boundary of a region of nucleosome-free chromatin, suggesting that ABF1 may affect local chromatin structure (65, 68). Furthermore, alterations in nucleosome position at *ARS1* have measurable effects on ARS activity (65). Correlations between the effects of ABF1 on chromatin structure and changes in ARS activity (or transcription control of adjacent genes) could readily be examined by using the *abf1*(Ts) strains. In addition, the development of in vitro DNA replication assays would be useful in determining whether ABF1 acts through a mechanism similar to that of viral replication or transcription factors.

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